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(54) Title: CHIMERIC MOLECULES CONTAINING A MODULE ABLE TO TARGET SPECIFIC CELLS AND A MODULE REGULATING THE APOPTOGENIC FUNCTION OF THE PERMEABILITY TRANSITION PORE COMPLEX (PTPC)

(57) Abstract: A chimeric polypeptide has the formula: pTox-pTarg, wherein pTox is a viral apoptotic peptide, such as the Vpr peptide of HIV-1 or a fragment of the Vpr peptide of HIV-1 containing the amino acid motif H(F/S)RIG that interacts with mitochondrial inner membrane, adenine nucleotide translocation (ANT) protein of a cell. pTarg is an antibody or an antibody fragment that binds to the outer membrane of the cell. Binding of the chimeric polypeptide to the cell is followed by apoptosis of the cell. A vector encoding a chimeric polypeptide and a recombinant host cell comprising the vector are provided. The chimeric polypeptide us useful for targeting pTox to cells, such as cancer cells.

CHIMERIC MOLECULES CONTAINING A MODULE ABLE TO TARGET SPECIFIC CELLS AND A MODULE REGULATING THE APOPTOGENIC FUNCTION OF THE PERMEABILITY TRANSITION PORE COMPLEX (PTPC)

CROSS-REFERENCE TO RELATED APPLICATIONS

The application hereby claims the benefit under 35 U.S.C. § 119(e) of United States provisional application Serial No. 60/265,594, filed February 2, 2001. The entire disclosure of this application is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cell death regulatory molecules for therapeutic use. More specifically, this invention relates to molecules in which a peptidic or pseudo-peptidic part acting on the permeability transition pore complex (PTPC) is covalently linked to cell-targeting molecules including antibodies, recombinant antibody fragments or homing peptides. The resulting chimeric molecules are polypeptides or peptidomimetic molecules which target the PTPC and/or its major component the adenine nucleotide translocation (ANT) to induce or inhibit cell death (apoptosis). This invention also relates to such chimeric molecules when the PTPC-interacting part is an apoptogenic HIV-1 Vpr-derived peptide (or pseudopeptide) or an ANT-derived peptide (or pseudo-peptide). This invention also relates to nucleic acid sequence construct encoding such chimeric molecule or encoding portions of these chimeric molecules.

Background

It is currently agreed that mitochondria play an important role in controlling life and death of cells (apoptosis; Kroemer and Reed 2000, Nature Medicine). It appears both that an increasing number of molecules involved in the transduction of the signal and also many metabolites and certain viral effectors act on mitochondria and influence the permeabilisation of mitochondrial membranes. Using mitochondrial-specific pro-apoptotic agent would seem to be an emerging concept in cancer therapy (Costantini et al 2000, Journal of the National Cancer Institute). Similarly, it might be possible to use cytoprotective molecules, thanks to their ability to stabilize mitochondrial membranes, in the treatment of illnesses where there is excessive apoptosis (neurodegenerative diseases, ischemia, AIDS, fulminant hepatitis, etc.).

Mitochondrial membrane permeabilisation (MMP) is a key event of apoptotic cell death associated with the release of caspase activators and caspase-independent death effectors from the intermembrane space, dissipation of the inner transmembrane potential ($\Delta\Psi m$), as well as a perturbation of oxidative phosphorylation (Green and Reed, 1998; Gross *et al.*, 1999; Kroemer and Reed, 2000; Kroemer *et al.*, 1997; Lemasters et al., 1998; Vander Heiden and Thompson, 1999; Wallace, 1999). Pro- and anti-apoptotic members of the Bcl-2 family regulate inner and outer MMP through interactions with the adenine nucleotide translocation (ANT; in the inner membrane, IM), the voltage-dependent anion channel (VDAC; in the outer membrane, OM), and/or through autonomous channel-forming activities (Desagher et *al.*, 1999; Gross et al., 1999; Kroemer and Reed, 2000; Marzo et al., 1998; Shimizu et al., 1999; Vander Heiden and Thompson, 1999). ANT and VDAC are major components of the permeability transition pore complex (PTPC), a polyprotein structure organized at sites at which the two mitochondrial membranes are apposed (Crompton, 1999; Kroemer and Reed, 2000).

The mitochondrial phase is under the control of Bcl-2 family of oncogenes and anti-oncogenes (for review: 5; 28) involved in more than 50% of cancers (29). All members of Bcl-2 family play an active role in the regulation of apoptosis, some of them being proapoptotic (Bax, Bak, Bcl- X_S, Bad, etc.) and others, being antiapoptotic (Bcl-2, Bcl-X_L, Bcl-w, Mcl-l, etc.) (G. Kroemer, Nat Med 3, 614-20 (1997)).

The mitochondrial megachannel is a polyprotein complex formed in the contact site between the inner and the outer mitochondrial membranes that participate in the regulation of mitochondrial membrane permeability. It is composed of a set of proteins including mitochondrion-associated hexokinase (HK), porin (voltage-dependent anion channel or VDAC), adenine nucleotide translocation (ANT), peripheral benzodiazepin receptor (PBR), creatine kinase (CK), and cyclophilin D, as well as Bcl-2 family members. In physiological conditions, PTPC controls the mitochondrial calcium homeostasis via the regulation of its conductance by the mitochondrial pH, the $\Delta\Psi m$, NAD/NAD(P)H redox equilibrium and matrix protein thiol oxidation. (M. Zoratti, I. Szabo, Biochim, Biophys Acta 1241, 139-76 (1995). S. Shimizu, M. Narita, Y. Tsujimoto, Nature 399, 483-487 (1999). M. Crompton, Biochem J 341, 233-249 (1999). K. Woodfield, A. Ruck, D. Brdiczka, A. P. Halestrap, Biochem J 336, 287-90 (1998).

P. Bernardi, K. M. Broekemeier, D. R. Pfeiffer, J Bioenerg Biomembr 26, 509-17 (1994). F. Ichas, L. Jouaville, J. Mazat, Cell 89, 1145-53 (1997)).

Apoptosis and related forms of controlled cell death are involved in a great number of illness. Excess or insufficiency of cell death processes are involved in auto-immune and neurodegenerative diseases, cancers, ischemia, and pathological infections or diseases such as viral and bacterial infections. Just few examples illustrating the virtually ubiquitous involvement of mitochondria in diseases associated with the abnormal control of cell death will be mentioned here.

In different models of ischemia (heart, liver, kidney or brain), using molecules that are capable of stabilising mitochondrial membranes, such as CsA for example (or its analogous non-immunosuppressor –Me-Val4-CsA) has made it possible to reduce massive apoptosis and its acute consequences at the level of the organ. In addition, VDAC is indispensable for the destruction of neurons of the rat hippocampus after hypoxic reperfusion. In the area of neurodegenerative diseases, a great many observations suggest close links with mitochondrial control of apoptosis (see Kroemer and Reed 2000, Nature Medicine). The neurotoxin –methyl-4-phenylpyridinium induces mitochondrial permeability transition and the exit of cytochrome c. Poisoning by mitochondrial toxins such as nitro-propionic acid or rotenone provokes in primates and rodents a Huntington-disease type of illness.

PTPC is a dynamic protein complex located at the contact site between the two mitochondrial membranes, its opening allowing the free diffusion of solutes < 1500 Da on the inner membrane. Formation of PTPC involves the association of proteins from different compartments, hexokinase (cytosol), porin, also called voltage-dependent anion channel (VDAC, outer membrane), peripheral benzodiazepin receptor (PBR, outer membrane), ANT (inner membrane) and cyclophilin D (matrix). PTPC has been implicated in many examples of apoptosis due to its capacity to integrate multiple pro-apoptotic signal transduction pathways and due to its control by proteins from Bcl-2/Bax family. The Bcl-2 family comprises death inhibitory (Bcl-2-like) and death inducing (Bax-like) members which respectively prevent or facilitate PTPC opening. Bax and Bcl-2 reportedly interact with VDAC and ANT within PTPC. In physiological conditions, ANT is a specific antiporter for ADP and ATP. However, ANT can also form a lethal pore upon interaction with different pro-apoptotic agents. including Ca2+,

atractyloside, HIV-1 Vpr-derived peptides and pro-oxidants. Mitochondrial membrane permeabilization may also be regulated by the non-specific VDAC pore modulated by Bcl-2/Bax-like proteins in the outer membrane (12; 16). and/or by changes in the metabolic ATP/ADP gradient between the mitochondrial matrix and the cytoplasm (17).

There is a need in the art for cytoprotective molecules in ischemia, neurodegenerative diseases, fulminant hepatitis and viral infections.

Another application of the chimeric molecule according the invention can be contemplated for the preparation of cosmetics or for preventing early death of plants or vegetables or flowers particularly for preventing the opening of the PTPC.

Conventional chemotherapeutic agents are limited in their therapeutic effectiveness by severe side effects due to their poor selectivity for tumors. The development of monoclonal antibodies (and ScFv) against specific tumor antigens and the identification of homing peptides specific for tumor vascularisation have made it possible to consider enhancing the selectivity of anticancer drugs by a targeted delivery approach. However, such reported attempts using monoclonal antibodies and the anticancer drugs doxorubicin (Trail P.A., et al 1993 Science 261:212), metotrexate (Kanellos J. et al., 1985 J Natl Cancer Inst 75:319), and Vinca alkaloids (Starling J.J. et al., 1991 Cancer Res 41:2965), have been largely unsuccessful. These antibody-drug conjugates were only moderately potent and usually less cytotoxic than the corresponding unconjugated drugs. In fact, antigen-specific cytotoxicity toward cultured tumor cells was rarely demonstrated. *In vivo* therapeutic effects with these conjugates in tumor zenograft animal models were in general observed only when the treatments were commenced before the tumors were well established or when exceedingly large doses (up to 90 mg/kg, drug equivalent dse) were used. It is, therefore, not surprising that in human clinical trials, no significant antitumor effects were observed with these agents (Elias D.J. et al., 1994 Am Respir Crit Care Med 150:1114) (Schneck D. et al., 1990). Indeed, the peak circulating serum concentrations of conjugates were only in the same range as their *in vitro* IC50 value and thus, capable of eliminating at best only about 50% of tumor cells.

These observations led to the conclusion that the previous attempts at delivering therapeutic doses of cytotoxic drugs via monoclonal antibodies have met with little success in clinical trials because of inappropriate choice of drug. One possible (partial-) solution was to

conclude that immunoconjugates must be composed of drugs possessing much higher potency than the clinically used anticancer agents if therapeutic levels of conjugate at the tumor sites are to be achieved in patients. Effectively, such toxins, including maytansinoides, enediynes, or intercalating agents CC1065, were shown to be 100 to 1000-fold more cyctotoxic than the chemotherapeutic agents doxorubicin, methotrexate, and Vinca alkaloids (Chari RVJ et al., 1995 Cancer Res 55:4079) (Chari RVJ et al., 1992, Cancer Res 52:127).

Another approach termed "Adept" was also designed. This antibody-directed enzyme prodrug therapy (Adept) is based upon the use of a monoclonal antibody to target an enzyme at the tumor cell surface, which ultimately is expected to selectively deliver an antitumor drug from a suitable inactive prodrug. In both cases, clinical trials are in progress; however, since today none of them have been introduced in cancer chemotherapy, there is a need for new tools to kill target tumor cells. Bagshawe KD, 1990. Antibody-directed enzyme/prodrug therapy (ADEPT). Biochem Soc Trans. 18(5):750-2. Melton RG, Sherwood RF. 1996 Antibody-enzyme conjugates for cancer therapy. J Natl Cancer Inst, 88(3-4):153-65. Rihova B. 1997; Targeting of drugs to cell surface receptors. Crit Rev Biotechnol. 17(2):149-69. Hudson PJ. 2000. Recombinant antibodies: a novel approach to cancer diagnosis and therapy. Expert Opin Investig Drugs 9(6):1231-42.

Recently, the mitochondrion has been proposed as a novel prospective target for chemotherapy-induced apoptosis (1-7). Indeed, four different anti-cancer agents, including the resinoid acid-derivative CD437, lonidamine, betulinic acid, and arsenite, have been shown to induce cancer cell apoptosis by a direct action on mitochondria. The interaction of these anti-cancer agents with mitochondria results in an increase of the permeability of the inner mitochondrial membrane due, at least in part, to the opening of the permeability transition pore complex (PTPC). PTPC opening leads to swelling of the mitochondria matrix, the dissipation of the inner transmembrane potential ($\Delta\Psi m$), enhanced generation of reactive oxygen species (ROS), and the release of apoptogenic proteins from the intermembrane space to the cytoplasm. Such mitochondrial apoptogenic effectors include the caspase activator cytochrome c, apoptosis inducing factor (AIF), and pro-caspases (2-6). All the signs of apoptosis induced by CD437, lonidamine, betulinic acid, and arsenite are prevented by two agents acting on specific PTPC proteins, namely cyclopsporin A (CsA, a cyclophilin D ligand) and bongkrekic acid (BA, a

ligand of the adenine nucleotide translocase (ANT)). It thus appears that PTPC opening is a critical event of apoptosis triggered by these agents.

Mastoparan, a peptide isolated from wasp venom, is the first peptide known to induce mitochondrial membrane permeabilization via a CsA-inhibitable mechanism and to induce apoptosis via a mitochondrial effect when added to intact cells. This peptide has an α-helical structure and possesses some positive charges that are distributed on one side of the helix. A similar peptide (KLAKLAKKLAKLAK or $(KLAKLAK)_2$ (K = lysine, L = alanine, and A = leucine) has been found recently to disrupt mitochondrial membranes when it is added to purified mitochondria, although the mechanisms of this effect have not been elucidated.

The vasculature of individual tissues is highly specialized. The endothelium in lymphoid tissues expresses tissue-specific receptors for lymphocyte homing, and recent work utilizing phage homing has revealed an unprecedented degree of specialization in the vasculature of other normal tissues. *In vivo* screening of libraries of phage that displace random peptide sequences on their surfaces has yielded specific homing peptides for a large number of normal tissues. The tissue-specific endothelial molecules to which the phage peptides home may serve as receptors for metastasizing malignant cells. Probing of tumor vasculature has yielded peptides that home to endothelial receptors expressed selectively in angiogenic neovasculature. These receptors, and those specific for the vasculature of individual normal tissues, are likely to be useful in targeting therapies to specific sites. Ruoslahti E, Rajotte D. 2000; An address system in the vasculature of normal tissues and tumors. Annu Rev Immunol. 18:813-27.

Ellerby et al. recently have fused the mitochondriotoxic $(KLAKLAK)_2$ motif to a targeting peptide that interacts with endothelial cells. Such a fusion peptide is internalized and induces mitochondrial membrane permeabilization in angiogenicendothelial cells and kills MDA-MD-435 breast cancer xenografts transplanted into nude mice. Similarly, a recombinant chimeric protein containing interleukin 2 (IL-2) protein fused to Bax selectively binds to and kills IL-2 receptor-bearing cells *in vitro*. Thus, specific cytotoxic agents that target surface receptors, translocate into the cytoplasm, and induce apoptosis via mitochondrial membrane permeabilization might be useful in treating cancer.

There is a need in the art for the selective eradication of transformed cells. One strategy is to target a toxic agent to selected cell types. More particularly, there exists a need in the art for method and reagents for regulating mitochondrial permeabilization and apoptosis.

Summary of the Invention

In order to overcome at least some of the limitations of the prior art, the present invention provides a peptidic or pseudo-peptidic family of polyfunctional molecules containing a cell-targeting part (termed TARG), a PTPC-interacting part (termed TOX/SAVE), and a facultative mitochondrial localisation sequence (MLS). In a preferred embodiment of the invention, the TOX/SAVE portion of the said polyfunctional molecule is a peptide or peptidomimetic molecule which interact directly with the Adenine Nucleotide Translocator (ANT) a central component of the PTPC

Thus, the present invention includes two categories of targeted cell death regulatory molecules:

- TARG-(MLS)-TOX is a polyfunctional molecule which induces a PTPC-dependent mitochondrial membrane permeabilisation and consequent cell death.
- TARG-(MLS)-SAVE is a polyfunctional molecule which protects cells from mitochondrial membrane permeabilisation and consequently from cell death through interaction with the PTPC and/or ANT.

The invention further provides a vector encoding a chimeric polypeptide of the invention.

Also, the invention provides a recombinant host cell comprising a vector of the invention.

Further, the invention provides a cancer cell having a tumor-associated antigen on the surface thereof to which the chimeric polypeptide of the invention is bound via the antibody or antibody fragment of the chimeric polypeptide. The invention also provides methods for detecting cancer cells.

The invention also provides methods for inducing or preventing apoptosis with polypeptides of the invention. The invention provides methods for inducing apoptosis in tumor cells. The invention provides methods for inducing apoptosis in virus infected cells.

The invention further provides hybridomas producing polypeptides of the invention. The invention also provides monoclonal antibodies produced by these hybridomas.

The invention also provides methods for identifying active agents of interest that interact with the PTPC. The invention also provides methods for identifying active agents of interest that interact with ANT peptide. The invention also provides methods for identifying mitochondrial antigens.

The invention also provides methods of treatment or prevention of a pathological infection or disease by administering a polypeptide of the invention to a patient. The invention also provides pharmaceutical compositions comprising a polypeptide of the invention.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of vector pACgp67-ScFv461.

Figure 2 shows the nucleotide sequence of vector pACgp67-ScFv350.

Figure 3 shows the nucleotide sequence of Vh and VL, from the clone therap 99B3.

Figure 4 shows the nucleotide sequence of Vh and VL from the clone therap.88E10.

Figure 5 shows the nucleotide sequence of Vh and VL from the clone therap.152C3.

Figure 6, 7, 8, 9, 10, 11 show surface plasmon resonance curves.

Figures 12 and 13 show the strategy for obtaining the ScFv-transfert vector.

Detailed Description of the Invention

It was recently discovered that the proapoptotic HIV-1-encoded protein Vpr induces mitochondrial membrane permeabilization via its physical and functional interaction with the mitochondrial inner membrane protein ANT (adenine nucleotide translocation, also called ADP/ATP carrier). This was shown using a variety of different techniques: surface plasmon resonance, electrophysiology, synthetic proteoliposomes, studies on purified mitochondria (respirometry, electron microscopy, organellofluorometry), as well as microinjection of intact cells. These discoveries are described in detail in U.S. Provisional Application No. 60/231,539 filed September 11, 2000, the entire disclosure of which is relied upon and incorporated by reference herein.

The present invention pertains to novel cytotoxic conjugates based on the association between a peptidic molecule (named pTox) interacting with the mitochondrial permeability transition pore complex (PTPC) and a molecule (named pTarg) able to target cells. The present

invention also pertains to novel cytoprotective conjugates based on the association between a peptidic molecule (named SAVE) interacting with the mitochondrial permeability transition pore complex (PTPC) and a molecule (named pTarg) able to target the cells to rescue. In a specific embodiment of this invention, a cytotoxic conjugate of the invention includes a viral derived pro-apoptotic peptide.

In one embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX is a tumor specific molecule that selectively interact with a tumor cell or a specific mammalian cell type, where the polyfunctional molecule is selectively internalised by the mammalian or tumoral cell type, where the polyfunctional molecule interact with the PTPC and/or ANT and exhibits thereto a strong mitochondrio-toxicity leading to apoptosis or any cell death process.

In one embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX exhibits a selective toxicity against angiogenic endothelial cells. In another embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX exhibits a selective toxicity against tumor cells.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is an antibody or a recombinant antibody fragment. In another embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is tumor horning peptide (example; CNGRC peptide; lung-homing peptide CGFECVRQCPERC).

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a peptide or a peptido-mimetic derived from the C-terminal part (amino-acids 52 to 96) of the HIV-1 Vpr protein.

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a pro-apoptotic Bcl-2 family member such as the Bax or Bid proteins, or a fragment thereof.

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a D-peptide, is a Ψ-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table 1:

Table I:

Name	TOX Peptidic Sequences
Vpr71-82	HFRIGCRHSRIG

Vpr71-82[R73,77,80K]	HFKIGCKHSKIG
Vpr71-96	HFRIGCRHSRIGIIQQRRTRNGASKS
Vpr71-96[R73,77,80K]	HFKIGCKHSKIGIIQQRRTRNGASKS
Vpr52-96	DTWTGVEALIRILQQLLFIHFRIGCRHSRIGIIQQRRTRNGASKS
Vpr52-96[R73,77,80K]	DTWTGVEALIRILQQLLFIHFKIGCKHSKIGIIQQRRTRNGASKS
Vpr52-96[L60,67A]	DTWTGVEAAIRILQQALFIHFRIGCRHSRIGIIQQRRTRNGASKS
Vpr52-82	DTWTGVEALIRILQQLLFIHFRIGCRHSRIG
Vpr52-82[R73,77,80K]	DTWTGVEALIRILQQLLFIHFKIGCKHSKIG
Histatin5 Candida Albicans	DSHARKRHHGYKRKFHEKHHSHRGY
Mastoparan Vespula Lewisii	INLKALAALAKKIL
hNUR77(555-568)	LSRLLGKLPELRTL
hNTR(368-381) neutrotrophin receptor	ATLDALLAALRRIQ
Bid(84-100)	RNIARHLAQVGDSMRDR
Bax(57-72)	KKLSECLKRIGDELDS
Bax(72-87)	GQVGRQLAIIGDDINR
HBX(70-78)	ALRFTSARR
DCC(1376-1390)	KTHVKTASLGLAGKA
ANT_1(104-116)	DRHKQFWRYFAGN
ANT_2(104-116)	DKRTQFWRYFAGN
ANT_3(104-116)	DKHTQFWRYFAGN
ANT_1(104-116 [A114P]	DRHKQFWRYFPGN
ANT_2(104-116)[A114P]	DKRTQFWRYFPGN
ANT_3(104-116)[A114P]	DKHTQFWRYFPGN
$ANT_{1,2,3}$(117-134)	LASGGAAGATSLCFVYPL
ANT_1(104-134)	DRHKQFWRYFAGNLASGGAAGATSLCFVYPL
ANT_2(104-134)	DKRTQFWRYFAGNLASGGAAGATSLCFVYPL
ANT_3(104-134)	DKHTQFWRYFAGNLASGGAAGATSLCFVYPL
ANT_1(104-134)[A114P]	DRHKQFWRYFPGNLASGGAAGATSLCFVYPL
ANT_2(104-134 [A114P]	DKRTQFWRYFPGNLASGGAAGATSLCFVYPL
ANT_3(104-134) [A114P]	DKHTQFWRYFPGNLASGGAAGATSLCFVYPL
Vpr 52-96 [C76S]	DTWTGVEALIRILQQLLFIHFRIGSRHSRIGIIQQRRTRNGASKS
HTLV-Ip13II	$_{19}$PSLRVWRLCARRLV$_{32}$

Bad103-127	NLWAAQRYGRELRRMSDEFVDSFKK
Bax52-76	QDASTKKLSECLKRIGDELDSNMEL

In one embodiment of the invention, the SAVE part of the polyfunctional molecule TARG-(MLS)-SAVE is a L-peptide, a D-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table II:

Name	SAVE Peptidic Sequences
ANT_1(104-116)	DRHKQFWRYFAGN
ANT_2(104-116)	DKRTQFWRYFAGN
ANT_3(104-116)	DKHTQFWRYFAGN
$ANT_{1,2,3}$(117-134)	LASGGAAGATSLCFVYPL
ANT_1(104-134)	DRHKQFWRYFAGNLASGGAAGATSLCFVYPL
ANT_2(104-134)	DKRTQFWRYFAGNLASGGAAGATSLCFVYPL
ANT_3(104-134)	DKHTQFWRYFAGNLASGGAAGATSLCFVYPL

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MIS)-SAVE is a L-peptide, a D-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table III:

ANTENNAPEDIA third helix (residues 43-58)	RQIKITFQNRRMKTKK
HIV-1 Vpr 83-96 transduction domain	IIQQRRTRNGASKS
HIV-1 Tat48-59 transduction domain	GRKKRRQRRRPP
HIV-1 Tat49-57 transduction domain	RKKRRQRRR
pep-1	KETWWETWWTEW

In one embodiment of the invention, the Targ part of the polyfunctionnal molecule TARG-(MLS)-TOX is the decanoic acid $CH_3(CH_2)_8CO$-.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is an antibody, a recombinant antibody, a recombinant antibody fragment or a ScFv (single chain fragment variable).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is encoded by the following vector pACgp67-ScFv461 (figure 1).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is encoded by the following vector pACgp67-ScFv350 (figure 2).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is a tumor homing peptide as defined by Ellerby et al in PCT/US00/01602.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX/SAVE is a brain or kidney homing peptide as defined by Pasqualini R, Ruoslahti (in Nature 1996 Mar 28;380(6572):364-6. Organ targeting in vivo using phage display peptide libraries).

In one embodiment of the invention, pTox is the Vpr peptide of HIV-1 or a fragment thereof. Protein R (Vpr) of human immunodeficiency virus type 1 (HIV-1) is a virion-associated viral gene product with an average length of 96 amino acids, and a molecular weight of approximately 15 kD. Vpr is a highly conserved viral protein among HIV, simian immunodeficiency viruses (SIV). *See* Yuqi Zhao and Robert T. Elder, "Yeast Perspectives on HIV-1 VPR," Frontiers in Bioscience 5, d905-916, December 1, 2000.

Vpr has been characterized as an oligomer, and is thought to be divided into three domains on the basis of its structural features: an amino-terminal, negatively charged region that is predicted to form an amphipathic α helix (amino acids 17 to 34); a central hydrophobic domain (amino acids 35 to 75); and a carboxy-terminal, positively charged domain (amino acids 80 to 96). Mutational analysis of Vpr suggests that the nuclear import, virion incorporation, and cell cycle arrest of Vpr are mediated by the distinct functional domains. A structural motif within an amino-terminal helix appears to be important for packaging of Vpr into virions and for maintaining the stability of the protein. A central hydrophobic region, especially the leucine-isoleucine (LR) domain, is reported to be involved in the nuclear localization of Vpr. The cell

cycle arrest function of Vpr was found to be largely located within a carboxy-terminal, positively charged region. *See* Tomoyuki Yamaguchi, Nobumoto Watanabe, Hiromitsu Nakauchi, and Atsushi Koito, "Human Immunodeficiency virus type 1 Vpr Modifies Cell Proliferation via Multiple Pathways," Microbiol, Immunol., 43(5), 437-447, 1999.

The amino acid sequence of human immunodeficiency virus type 1 viral protein R (Vpr) is shown below:

MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYE
TYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS.

Vpr and peptides containing conserved H(F/S)RIG repeat motifs can rapidly penetrate human CD4 cells, and cause mitochondrial dysfunction and death by apoptosis. More particularly, recombinant Vpr and C-terminal peptides of Vpr containing the conserved sequence HFRIGCRHSRIG can cause permeabilization of $CD4^+$ T lymphocytes, a dramatic reduction of mitochondrial membrane potential, and finally cell death. Vpr and Vpr peptides containing the conserved sequence rapidly penetrate cells, co-localize with the DNA, and cause increased granularity and formation of dense apoptotic bodies. Vpr treated cells undergo apoptosis, and this was confirmed by demonstration of DNA fragmentation. *See* C. Arunagiri, I. Macreadie, D. Hewish and A. Azad, "A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human $CD4^+$ lymphocytes," Apoptosis 1997; 2: 69-76.

Using a yeast model system, it has been confirmed that there is a cytocidal activity associated with the C-terminal portion of Vpr, particularly the sequence HFRIGCRHSRIG. Vpr and portions of Vpr containing the sequence HFRIGCRHSRIG can kill a range of mammalian cells including human lymphocytes. *See* I.G. Macreadie, A, Kirkpatrick, P.M. Strike, and A.A. Azad, "Cytocidal Activities of HIV-1 VPR and Sac1p peptides Bioassayed in Yeast," Protein and Peptide Letters, Vol. 4, No. 3, pp. 181-186, 1997.

The C-terminal moiety (Vpr52-96), within an α-helical motif of 12 amino acids (Vpr71-82), contain several critical arginine (R) residues (R73, R77, R80), which are strongly conserved among different pathogenic HIV-1 isolates. L.G. Macreadie, et al., Proc. Natl. Acad. Sci. USA 92, 2770-2774 (1995). I.G. Macreadie, et al., FEBS Lett. 410, 145-149 (1997). E. Jacotot, et al., J. Exp. Med. 191, 33-45 (2000). Thus, the pro-apoptotic portion (pTox) of the chimeric

polypeptide of the invention can contain, for example, the sequence HFRIGCRHSRIG (HIV-1 Vpr71-82), HFKIGCKHSKIG, Vpr 71-96, Vpr 52-96, or a pseudo peptidic variant such as D[HFRIGCRHSRIG].

Other variants of Vpr peptides can also be employed in this invention. Peptide fragments of Vpr encompassing a pair of H(F/S)RIG sequence motifs (residues 71-75 and 78-82 of HIV-1 Vpr) have been shown cause cell membrane permeabilization and death in yeast and mammalian cells. Peptide Vpr $^{59-86}$ (residues 59-86 of Vpr) forms an α-helix encompassing residues 60-77, with a kink in the vicinity of residue 62. It has been shown that the first of the repeated sequence motifs (HFRIG) participates in a well-defined α-helical domain, whereas the second (HSRIG) lay outside the helical domain and forms a reverse turn followed by a less ordered region. On the other hand, peptides Vpr^{71-82} and Vpr^{71-96}, in which the sequence motifs are located at the N-terminus, were largely unstructured under similar conditions, as judged by their C^2H chemical shifts. Thus, it has been shown that the HFRIG and HSRIG motifs adopt α-helical and turn structures, respectively, when preceded by a helical structure, but are largely unstructured in isolation. There are implications of these findings for interpretation of the structure-function relationships of synthetic peptides containing these motifs. For example, since the HFRIG and HSRIG sequence motifs adopt helical and turn structures, respectively, when preceded by a helical structure, as in full-length Vpr, but are largely unstructured in isolation, 7-8 residues, sufficient to support at least 1-2 turns of helix, should be included at the N-terminus of Vpr when used as the pTox component of the chimeric polypeptides of the invention to ensure that they are able to adopt the same structure as in the full-length protein. *See* Shenggen Yao, Allan M. Torres, Ahmed A. Azad, Ian G. Macreadie and Raymond S. Norton, "Solution Structure of Peptides from HIV-1 Vpr Protein that Cause Membrane Permeabilization and Growth Arrest," J. Peptide Sci. 4: 426-435 (1998). While the Vpr gene codes for a protein of 96-amino-acids, variations have been observed, e.g., Vprs from HIV-1_{HXB2} have 97 and 90-amino-acid residues, respectively. It will be understood that these variants can also be employed in this invention.

For the most effective toxicity, HFRIGCRHSRIG should be surrounded on each side by about eight amino acids from the native sequence. Vpr polypeptides and peptides of greater than 9 amino acids that inhibit or augment Vpr binding, mitochondrial membrane permeabilization, or apoptosis can also be employed in the invention, as well as peptides that are at least 10-20, 20-

30, 30-50, 50-100, and 100-365 amino acids in size. DNA fragments encoding these polypeptides and peptides are encompassed by the invention. Flanking residues should not disrupt the helical structures described above.

The Vpr variants and other viral apoptotic peptides can be assessed for their ability to mediate apoptosis, and thus their suitability for use as pTox in the invention. It is understood that many techniques could be used to assess binding of Vpr or another viral apoptotic peptide to ANT, and that these embodiments in no way limit the scope of the invention. For example, in one embodiment, surface plasmon resonance is used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, electrophysiology is used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, purified mitochondria are used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, synthetic proteoliposomes are used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, microinjection of live cells is used to assess binding of Vpr or another viral apoptotic peptide to ANT. These techniques are described in U.S. Provisional Application No. 60/231,539.

In another embodiment, the yeast two-hybrid system developed at SUNY (described in U.S. Patent No. 5,282,173 to Fields et al.; J. Luban and S. Goff., *Curr Opin. Biotechnol.* 6:59-64, 1995; R. Brachmann and J. Boeke, *Curr Opin. Biotechnol.* 8:561-568, 1997; R. Brent and R. Finley, *Ann. Rev. Genet.* 31:663-704, 1997; P. Bartel and S. Fields, *Methods Enzymol.* 254:241-263, 1995) can be used to screen for Vpr-ANT interaction as follows. Vpr, or portions thereof, or another viral apoptotic peptide, responsible for interaction, can be fused to the Gal4 DNA binding domain and introduced, together with an ANT molecule fused to the GAL 4 transcriptional activation domain, into a strain that depends on GAl4 activity for growth on plates lacking histidine. Interaction of the Vpr polypeptide or another viral apoptotic peptide with an ANT molecule allows growth of the yeast containing both molecules and allows screening for the molecules that inhibit or alter this interaction (i.e., by inhibiting or augmenting growth). In an alternative embodiment, a detectable marker (e.g. β-galactosidase) can be used to measure binding in a yeast two-hybrid assay.

Alternatively, the binding properties of Vpr peptide fragments or another viral apoptotic peptide can be determined by analyzing the binding of Vpr peptide fragments or another viral

apoptotic peptide to ANT-expressing cells by FACS analysis. This allows the characterization of the binding of the peptides, and the discrimination of relative abilities of the peptide to bind to ANT. *In vitro* binding assays with Vpr or another viral apoptotic peptide can similarly be used to characterize ANT binding activity.

In another specific embodiment, a cytotoxic conjugate of the invention includes an adenine nucleotide translocation (ANT)-derived pro-apoptotic peptide. The pro-apoptotic portion (pTox) of the conjugate can contain, for example, the sequence DKRTQFWRYFPGN ($hANT_2$104-116[A114P]) or a pseudo-peptidic variant such as [DKRTQFWRYFPGN].

In another specific embodiment, a cytoprotective conjugate of the invention includes ANT-derived anti-apoptotic peptides. The anti-apoptotic portion (pSave) of the conjugate can contain, for example, the sequence DKRTQFWRYFAGN ($hANT_2$104-116), the sequence LASGGAAGATSLCFVYPL (ANT 117-134) or a pseudo-peptidic variant such as D[DKRTQFWRYFPGN].

The pTarg component of the chimeric polypeptide of the invention can be an antibody or an antibody fragment. The antibody or antibody fragment can be all or part of a polyclonal or monoclonal antibody. The term "antibodies" is meant to include polyclonal antibodies, monoclonal antibodies, fragments thereof, as well as any recombinantly produced binding partners. Antibodies are defined to be specifically binding if they bind with a K_a or greater than or equal to about 10^7 M^{-1}. Affinities of binding partners or antibodies can be readily determined using conventional techniques, for example those described by Scatchard *et al.*, *Ann. N.Y. Acad. Sci.*, 51:660 (1949).

As used herein, the term "antibody fragment" includes the following:

Fc	A constant region dimer lacking C_H1
Fab	A light chain dimerized to V_H-C_H1 resulting from papain cleavage; this is monomeric since papain cuts above the hinge cystines
F(ab)'$_2$	A dimer of Fab' resulting from pepsin cleavage below the hinge disulfides; this is bivalent and can precipitate antigen

Fab'	A monomer resulting from mild reduction of $F(ab)'_2$: an Fab with part of the hinge
Fd	The heavy chain portion of Fab (V_H-C_H1) obtained following reductive denaturation of Fab
Fv	The variable part of Fab: a V_H-V_L dimer
Fb	The constant part of Fab: a C_H 1-C_L dimer
pFc'	A C_H3 dimer

Fragments of monoclonal antibodies are of particular interest as small antigen targeting molecules. Antibody fragments are also useful for the assembly of the chimeric polypeptides of the invention designed to carry other pTox agents, such as a therapeutic conjugate. For *in vivo* applications, fragments of antibodies are of interest due to their altered pharmacokinetic behavior, which is useful for cancer therapy with cytotoxic agents, and for their rapid penetration into body tissues, which offer advantages for therapy techniques.

An antibody fragment of particular interest for use in the invention is a minimal Fv fragment with antigen-binding activity. The two chains of the Fv fragment are less stably associated than the Fd and light chain of the Fab fragment with no covalent bond and less non-covalent interaction, but nevertheless functional Fv fragments have been expressed for a number of different antibodies. Two strategies can be employed to stabilize the Fv fragments used in the invention: firstly, mutating a selected residue on each of the V_H and V_L chains to a cysteine to allow formation of a disulphide bond between the two domains; and secondly, the introduction of a peptide linker between the C-terminus of one domain and the N-terminus of the other, such that the Fv is produced as a single polypeptide chain known as a single-chain Fv.

Thus, single-chain Fvs (ScFvs), recombinant V_L and V_H fragments covalently tethered together by a polypeptide link and forming one polypeptide chain, are useful in this invention. For expression of Fv genes, several systems can be effectively used, including myeloma cells, insect, yeast, and *Escherichia coli* cells. Expression in *E. coli* has been a frequently used production method, with both intracellular expression and secretion enabling high yields of ScFv to be made.

The production of ScFv molecules requires the identification of a suitable peptide linker to span the 35-40 Å distance between the C-terminus of one domain and the N-terminus of the other and allow correct folding and assembly of the Fv structure. Several different types of linkers have been used and shown to result in functional ScFv. Polypeptides with the average length of 3-18 amino acids are usually used as links. They can be rich in serine and/or glycine residues, which introduce flexibility, or in charged glutamic acid and/or lysine residues, which improve solubility. Linkers can be selected from searching existing protein structures for protein fragments of the appropriate length and conformation, or by designing them *de novo* based on simple, flexible structures, such as the 15 amino acid sequence $(Gly_4Ser)_3$.

Active single-chain Fv molecules in both of the two possible orientations, V_H–linker–V_L or V_L–linker–V_H are useful in the invention; however, for some antibodies one particular orientation may be preferable as a free N-terminus of one domain, or C-terminus of the other, may be required to retain the native conformation and thus full antigen binding.

The ScFv may be susceptible to aggregation, with dimers, trimers, and multimers formed. The potential of forming dimers or other multimers with very short linkers, or no linker at all, can be exploited to produce stable pTarg structures. Such an approach can also be used to create pTarg molecules with two different binding specificities by fusing the V_H of an antibody of one specificity to the V_L of another and vice versa.

Fv's stabilized by disulphide linkages can also be employed as the pTarg component of the chimeric polypeptide of the invention. The introduction of a disulphide bond between the V_H and V_L domains to form a disulphide-linked Fv requires the identification of residues in close proximity on each chain, which are unlikely to affect directly the conformation of the binding site when mutated to cysteine, and will be capable of forming a disulphide bond without introducing strain into the structure of the Fv. Sites have been identified in both CDR regions and framework regions, which appear to result in the formation of such disulphide bonds and allow the production of stabilized Fv fragments which retain antigen-binding characteristics.

Due to small size, rapid clearance *in vivo*, stability, and easy engineering, ScFvs employed in this invention have various applications in the treatment of diseases, particularly of cancer. ScFvs can exhibit the same affinity and specificity for antigen as monoclonal antibodies. Dozens of ScFvs with different specificities have been constructed. They are useful for genetic

fusion to the potent toxins (pTox). If the monovalency of ScFv is a disadvantage, constructs with di- or multivalency with increased combining efficiency can be employed.

In a preferred embodiment of the invention, the targeting part (pTarg) of the cytotoxic conjugate is a recombinant portion (ScFv) of a tumor specific antibody, such as the ScFv versions of the M350 and V461 monoclonal antibodies. The hybridoma has been deposited at the CNCM on January 24, 2001, under the Accession Number I-2617.

The pTarg component of the chimeric polypeptide of the invention is preferably a monoclonal antibody or a fragment thereof. Monoclonal antibodies to human cell antigens are preferred. Many tumor-associated antigens are now known and characterized, and antibodies to these allow targeting to different tumor types. Useful tumor-associated antigens are absent on normal tissues and present at high levels on tumor cells, preferably homogeneously on all cells of the tumor. Antigen should also not be shed from the tumor into the blood.

Commonly used tumor-associated antigens and examples of antibodies raised against them are described in the following Table.

Antigen	Tumor type	Representative antibody
Turmor-associated glycoprotein 72 (TAG72), 72 kDa glycoprotein	Pancarcinoma	B72.3, CC49
Carcinoembryonic antigen (CEA), 180 kDa blycoprotein	Pancarcinoma	NP-4, A5B7
Polymorphic epithelial mucin (PEM), >100 kDa glycoprotein	Ovarian, breast, lung	HMFG1
Epithelial membrane antigen (EMA), 40 kDa glycoprotein	Colorectal (and other epithelial tumors)	17-1A
epidermal growth factor receptor (EGFR), 175 kDa glycoprotein	Breast, lung	425
$p185^{HER2}$/c-erb-B2		

Antigen	Tumor type	Representative antibody
(185 kDa glycoprotein)	Breast, lung	4D5
Prostate-specific membrane antigen (PSMA), 100 kDa glycoprotein	Prostrate	7E11-C5.3
CD33 67 kDa glycoprotein	Myeloid leukemia	P67.6,M195
CD 20 35 kDa glycoprotein	Lymphoma	C2B8
GD2 ganglioside	Melanoma, neuroblastoma	14-18

An important consideration is the absolute amount of antibody localized to the tumor site. Therefore, the ideal molecule would localize to the tumor in large amounts, delivering a high dose of pTox while clearing rapidly from the circulation and the rest of the body, minimizing non-specific toxicity. Intact antibodies typically circulate for a long period of time and accumulate high levels of activity at the tumor site, whereas antibody fragments clear more rapidly, sparing the dose to normal tissues.

The antibody fragments can also be prepared by phage-display technology. Phage display is a selection technique, according to which an antibody fragment (ScFv) is expressed on the surface of the filamentous phage fd. For this, the coding sequence of the antibody variable genes is fused with the gene that encoded the minor coat phage protein III (g3p) located at the end of the phage particle. The fused antibody fragments are displayed on the virion surface and particles with the fragments can be selected by adsorption on insolubilized antigen (panning). The selected particles are used after elution to reinfect bacterial cells. The repeated rounds of adsorbtion and infection lead to enrichment. Bacterial proteases can cleave the bond between the g3p protein and antibody fragments, which results in the production of soluble antibody fragments by infected bacterial cells. To release the soluble ScFvs, an excision of the g3p gene is made or an amber stop codon between the antibody gene and the g3p gene is engineered.

Immunoglobins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler *et al.*, P.N.A.S. USA 77:2197 (1980); Raso *et al.*, Cancer Res. 41:2073 (1981); Morrison *et al.*, Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison *et al.*, P.N.A.S. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams *et al.*, Biochemistry 19:2711-2719 (1980); Gough *et al.*, Biochemistry 19:2702-2710 (1980); Dolby *et al.*, P.N.A.S. USA, 77:6027-6031 (1980); Rice *et al.*, P.N.A.S. USA 79:7862-7865 (1982); Falkner *et al.*, Nature 298:286-288 (1982); and Morrison *et al.*, Ann. Rev. Immunol. 2:239-256 (1984). These materials and techniques can be employed to synthesize the pTarg component of the chimeric polypeptide of the invention.

Polyclonal antibodies employed as the pTarg component of the chimeric polypeptide of the invention can be readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats, using procedures that are well known in the art. In general, purified cell surface proteins or glycoproteins or a peptide based on the amino acid sequence of cell surface proteins or glycoproteins that is appropriately conjugated is administered to the host animal typically through parenteral injection. The immunogenicity of cell surface proteins or glycoproteins can be enhanced through the use of an adjuvant, for example, Freund's complete or incomplete adjuvant. Following booster immunizations, small samples of serum are collected and tested for reactivity to cell surface proteins or glycoproteins. Examples of various assays useful for such determination include those described in *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures, such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immunosorbent assays (ELISA), dot blot assays, and sandwich assays. See U.S. Patent Nos. 4,376,110 and 4,486,530.

Monoclonal antibodies employed as the pTarg component can be readily prepared using well known procedures. See, for example, the procedures described in U.S. Patent Nos. RE

32,011, 4,902,614, 4,543,439, and 4,411,993; Monoclonal Antibodies, Hybridomas: *A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980. Briefly, the host animals, such as mice, are injected intraperitoneally at least once and preferably at least twice at about 3 week intervals with isolated and purified cell surface proteins or glycoproteins, conjugated cell surface proteins or glycoproteins, optionally in the presence of adjuvant. Mouse sera are then assayed by conventional dot blot technique or antibody capture (ABC) to determine which animal is best to fuse. Approximately two to three weeks later, the mice are given an intravenous boost of cell surface proteins or glycoproteins or conjugated cell surface proteins or glycoproteins. Mice are later sacrificed and spleen cells fused with commercially available myeloma cells, such as Ag8.653 (ATCC), following established protocols. Briefly, the myeloma cells are washed several times in media and fused to mouse spleen cells at a ratio of about three spleen cells to one myeloma cell. The fusing agent can be any suitable agent used in the art, for example, polyethylene glycol (PEG). Fusion is plated out in plates containing media that allows for the selective growth of the fused cells. The fused cells can then be allowed to grow for approximately eight days. Supernatants from resultant hybridomas are collected and added to a plate that is first coated with goat anti-mouse Ig. Following washes, a label, such as ^{125}I-labeled cell surface proteins or glycoproteins, is added to each well followed by incubation. Positive wells can be subsequently detected by autoradiography. Positive clones can be grown in bulk culture and supernatants are subsequently purified over a Protein A column (Pharmacia).

The monoclonal antibodies for the pTarg component can be produced using alternative techniques, such as those described by Alting-Mees *et al.*, "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas", *Strategies in Molecular Biology* 3:1-9 (1990), which is incorporated herein by reference. Similarly, binding partners can be constructed using recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specific binding antibody. Such a technique is described in Larrick *et al.*, *Biotechnology*, 7:394 (1989).

The monoclonal antibodies and fragments thereof employed as the pTarg component include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, the

humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann *et al.* (*Nature* 332:323, 1988), Liu *et al.* (*PNAS* 84:3439, 1987), Larrick *et al.* (*Bio/Technology* 7:934, 1989), and Winter and Harris (*TIPS* 14:139, May 1993). Procedures to generate antibodies transgenically can be found in GB 2,272,440, US Patent Nos. 5,569,825 and 5,545,806 and related patents claiming priority therefrom, all of which are incorporated by reference herein.

In a further embodiment of the invention, the targeting part (pTarg) of a cytotoxic chimeric polypeptide is a tumor homing peptide. Such a tumor homing peptide include any homing sequence described by Ellerby *et al.*, in example V, VI, VII, VIII of PCT/US00/01602, the entire disclosure of which is relied upon and incorporated by reference herein.

In preferred embodiments of the invention, the chimeric polypeptide has the sequence CNGRCGG-HFRIGCRHSRIG, or CNGRCGG-D[HFRIGCRHSRIG], or CNGRCGG-Vpr52-96, or CNGRCGG-DKRTQFWYFPGN, or CNGRCGG-D[DKRTQFWYFPGN], or ACDCRGDCFCGG-HFRIGCRHSRIG, or ACDCRGDCFCGG-D[HFRIGCRHSRIG], or ACDCRGDCFCGG-Vpr52-96, or ACDCRGDCFCGG-DKRTQFWYFPGN, or ACDCRGDCFCGG-[DKRTQFWYFPGN], or M350/ScFv-HFRIGCRHSRIG, or M350/ScFv-D[HFRIGCRHSRIG] or M350/ScFv-Vpr52-96, or M350/ScFv-DKRTQFWYFPGN, or or M350/ScFv- D[DKRTQFWYFPGN].

Chimeric polypeptides of the invention can be generated by a variety of conventional techniques. Such techniques include those described in B. Merrifield, Methods Enzymol, 289:3-13, 1997; H. Ball and P. Mascagni, Int. J. Pept. Protein Res. 48:31-47, 1996; F. Molina *et al.*, Pept. Res. 9:151-155, 1996; J. Fox, Mol. Biotechnol. 3:249-258, 1995; and P. Lepage *et al.*, Anal. Biochem. 213: 40-48, 1993.

Peptides can be synthesized on a multi-channel peptide synthesizer using classical Fmoc-based and pseudopeptide synthesis. In one embodiment of the invention, Vpr52-96, Vpr71-96 and Vpr 71-82 and all the Tox, Save and TARG peptides described in Table I, II, III, are

synthesized by solid phase peptide chemistry. After cleavage from the resin, the peptides are purified and analyzed by reverse-phase HPLC. The purity of the peptides is typically above 98% according to HPLC trace. The integrity of each peptide can be controlled by matrix Assisted Laser Desorption Time of Flight spectrometry. To avoid rapid degradation of the peptides in biological fluids, one or several amide bonds could be advantageously replaced by peptide bond isosters like retro-inverso (NH-CO), methylene amino (CH_2-NH), carba (CH_2-CH_2) or carbaza (CH_2-CH_2-N(R)) bonds.

Alternatively, the chimeric polypeptides of the invention can be prepared by subcloning a DNA sequence encoding a desired peptide sequence into an expression vector for the production of the desired peptide. The DNA sequence encoding the peptide is advantageously fused to a sequence encoding a suitable leader or signal peptide. Alternatively, the DNA fragment may be chemically synthesized using conventional techniques. The DNA fragment can also be produced by restriction endonuclease digestion of a clone of, for example HIV-1, DNA using known restriction enzymes (New England Biolabs 1997 Catalog, Stratagene 1997 Catalog, Promega 1997 Catalog) and isolated by conventional means, such as by agarose gel electrophoresis.

In another embodiment, the well known polymerase chain reaction (PCR) procedure can be employed to isolate and amplify a DNA sequence encoding the desired protein or peptide fragment. Oligonucleotides that define the desired termini of the DNA fragment are employed as 5' and 3' primers. The oligonucleotides can contain recognition sites for restriction endonucleases, to facilitate insertion of the amplified DNA fragment into an expression vector. PCR techniques are described in Saiki *et al.*, Science 239:487 (1988); Recombinant DNA Methology, Wu *et al.*, eds., Academic Press, Inc., San Diego (1989), p. 189-196; and PCR Protocols: A Guide to Methods and Applications, Innis *et al.*, eds, Academic Press., (1990). It is understood of course that many techniques could be used to prepare polypeptide and DNA fragments, and that this embodiment in no way limits the scope of the invention.

Several methods can be used to link TARG to TOX and TARG to SAVE, depending on the particular chemical characteristics of the molecules. For example, methods of linking haptens to carrier proteins as used routinely in the field of applied immunology. In one embodiment, a premade a PTPC regulatory molecule (TOX or SAVE) can be conjugated to an antibody as antibody fragment (pTarg) using, for example, carbodiimide conjugation.

Carbodiimides comprise a group of compounds that have the general formula R-N+C=N-R, where R and R can be aliphatic or aromatic, and are used for synthesis of peptide bonds. The preparative procedure is simple, relatively fast, and is carried out under mild conditions. Cardodiimide compounds attack carboxylic groups to change them into reactive sites for free amino groups. Carbondiimide conjugation has been used to conjugate a variety of compounds for the production of antibodies.

The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) can be useful for conjugating a PTPC regulatory molecule (TOX or SAVE) to an antibody or antibody fragment molecule. Such conjugation requires the presence of an amino group, which can be provided, for example, by a PTPC regulatory molecule (TOX or SAVE), and a carboxyl group, which can be provided by an antibody or antibody fragment.

In addition to using carbodiimides for the direct formation of peptide bonds, EDC also can be used to prepare active esters, such as N-hydroxysucinimide (NHS) ester. The NHS ester, which binds only to amino groups, then can be used to induce the formation of an amide bond with the single amino group of the oxorubicin. The use of EDC and NHS in combination is commonly used for conjugation in order to increase yield of conjugate formation.

Other methods for conjugating a PTPC regulatory molecule (TOX or SAVE) to an antibody or antibody fragment also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can glutaraldehyde crosslinking. However, it is recognized that, regardless of which method of producing a chimeric polypeptide of the invention is selected, a determination must be made that an antibody or antibody fragment maintains its targeting ability and that a PTPC regulatory molecule (TOX or SAVE) maintains its activity.

The chimeric polypeptide of the invention may further incorporate a specifically non-cleavable or cleavable linker peptide functionally interposed between the PTPC regulatory molecule (TOX or SAVE) (pTarg) and the antibody or antibody fragment (pTox). Such a linker peptide provides by its inclusion in the chimeric construct, a site within the resulting chimeric polypeptide that may be cleaved in a manner to separate the intact PTPC regulatory molecule (TOX or SAVE) from the intact antibody or antibody fragment. Such a linker peptide may be, for instance, a peptide sensitive to thrombin cleavage, factor X cleavage, or other peptidase

cleavage. Alternatively, where the chimeric polypeptide lacks methionine, the antibody or antibody fragment may be separated by a peptide sensitive to cyanogen bromide treatment. In general, such a linker peptide will describe a site, which is uniquely found within the linker peptide, and is not found at any location in either of the TARG, TOX or SAVE fragment constituting the chimeric polypeptide.

Compositions comprising an effective amount of a chimeric polypeptide of the present invention, in combination with other components, such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. The chimeric polypeptide can be formulated according to known methods used to prepare pharmaceutically useful compositions. They can be combined in admixture, either as the sole active material or with other known materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can be complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance, and are thus chosen according to the intended application.

The compositions of the invention comprising the chimeric polypeptide can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration is performed according to art-accepted practices.

Compositions comprising nucleic acids in physiologically acceptable formulations are also contemplated. DNA may be formulated for injection, for example.

In one of its most general applications, the invention relates to a recombinant vector incorporating a DNA segment having a sequence encoding the chimeric polypeptide of the invention. For the purposes of the invention, the term "chimeric polypeptide" is defined as including any polypeptide where at least a portion of a viral apoptotic peptide is coupled to at least a portion of an antibody or antibody fragment. The coupling can be achieved in a manner that provides for a functional transcribing and translating of the DNA segment and message derived therefrom, respectively.

The vectors of the invention will generally be constructed such that the chimeric polypeptide encoding sequence is positioned adjacent to and under the control of an effective promoter. In certain cases, the promotor will comprise a prokaryotic promoter where the vector is being adapted for expression in a prokaryotic host. In other cases, the promoter will comprise a eukaryotic promoter where the vector is being adapted for expression in a eukaryotic host. In the later cases, the vector will typically further include a polyadenylation signal position 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the encoded chimeric polypeptide. Promoters of particular utility in the vectors of the invention are cytomegalovirus promoters and baculovirus promoters, depending upon the cell used for expression. Regardless of the exact nature of the vector's promoters, the recombinant vectors of the invention will incorporate a DNA segment as defined below.

A recombinant host cell is also claimed herein, which incorporates a vector of the invention. The recombinant host cell may be either a eukaryotic cell or a prokaryotic host cell. Where a eukaryotic cell is used, a Chinese Hamster Ovary (CHO) cell has utility. In another embodiment, when used in combination with a baculovirus promoter, the insect cell lines SF9 or SF21 can be used.

This invention will be described in greater detail in the following Examples.

EXAMPLE 1

Obtaining the murine monoclonal antibody (Ac M350)

Human fetal cells were chosen as a source of immunization. It was the well-known similarities between fetal and tumoral antigens which inspired us to use fetal cells as a source of immunization to produce monoclonal antibodies directed against the epitopes present on tumoral cells. Oncofetal antigens are glycoproteins which are present during intra-uterine life; they disappear at birth and can be re-expressed in pathological situations, particularly in malignant tumors. There are many examples of this antigen community, the best known models being fetoprotein which is associated with 70% of liver tumors, and <<embryo tumor antigens>>, which is often used in human clinical practice and which is a monitoring parameter for patients suffering from cancers of the digestive tract.

A. M350 clone production

These fetal cells were obtained from the sterile removal of the mammary buds of 25-week old female fetuses. Once the buds had been mechanically dissociated into 0.5 mm^3 fragments, the cells were resuspended in a Dulbecco medium modified with collagenase and hyalurodinase at 37°C and shaken for between 30 minutes and 4 hours after being monitored under the microscope. As soon as organoids appear, the cells were deposited onto Ficoll, washed, then cultured in a calcium-free DMEM-F12 medium, in hepes, insulin, choleric toxin, cortisol. Once the cells were subcultured once a week. Using this technique the cells duplicated 10 to 20 times giving sufficient cells for immunization purposes.

Balb/c mice were immunized four times, intraperiotonaly. The fusion was achieved according the classical technic of Kohler and Milstein. The screening was done with fetal mammary cells, adult mammary cells and breast tumors. Several clones appeared and one, M350 clone, was particularly tested on breast tumors and normal breast tissues. 150 tumor sections were tested: (i.e.) infiltrating intra-canalar and intra-lobular adenocarcimonas, infiltrating lobular adenocarcimonas. Tests were performed using an immunoenzymatic technic with alkaline phosphatase. All the tumors tested positive whereas the normal tissues taken from mammary

samples tested in parallel were negative for weakly positive. Each slide of normal tissue contained lobular type epithelial structures and cavities inside the paleal tissue.

B. Other Hybridomes

Obtaining new murine monoclonal antibodies against associated breast tumor antigens.

In this technology, C57/B16 mice were immunized four times, intraperitonaly, with a mixture of three different breast tumor cell lines (MCF7, MDA, ZR75-1). After fusion and screening the specificity was studied on normal breast tissues and malignant tumors, other tumor samples and peripheral blood cells. The Monoclonal antibodies showing surface tumor labeling were chosen.

<u>EXAMPLE 2</u>

A Cell lines and viruses

The insert cells derived from ovarian tissue of Spodoptera frugiperda (Sf9 insect cells, Vaughn et coll., 1977) and insect cells derived from Trichoplusia ni (High Five insect cells) were maintained at 28°C in TC100 medium supplemented with 5% fetal calf serum and were used for the propagation of recombinant baculoviruses and for the production of recombinant proteins. The recombinant baculoviruses are obtained after co-transfection of insect cells with baculovirus viral DNA (Baculogold, Pharmingen) and recombinant transfer vector DNA.

B. Recombinant transfer vector: pVL-PS-gp671

The recombinant transfer vector pVL-PSgp671 derived from transfer vector pVL1392 (Invitrogen) is used as transfer vector to generate recombinant viruses. It includes from 5' to 3' : the peptide signal sequence of gp67 baculovirus glycoprotein, the sequence coding for a His(6)-Tag, the recognition sequence for the Xa Factor, a polylinker region for subcloning the scFv sequence, a link-sequence: GGC required for the covalent association between cytotoxic peptides and ScFv.

The signal peptide sequence from gp67 was added by insertion of a PCR product of gp67 (obtained by PCR from a commercial pcGP67-B plasmid as a template and the PSgp67-Back and PSgp67-For as primers) at the *Bgl*II site of the pVL1392 plasmid. The sequence coding for the His(6)-Tag sequence and the recognition sequence for the Xa factor were then added by using

insertion of oligonucleotides at the 3' end of the gp67 sequence. By the same way the sequence of the peptide motif required for the covalent association between cytotoxic peptides and ScFv: (-Gly-Gly-Cys) was added at the 3' part of the polylinker (the first G is encoded by the last nucleotide of the Xmal site).

Insertion at BamH1 and Bg1I of overlaping primers:

Th1: GAT CCC ATC ATC ACC ACC ACC AC (BamHI-His(6))

Th2: ATT GAA GGA AGA GAATTC CCATG (Factor Xa cleveage -EcoRI-NcoI)

Th3: GCT GCA GCC CGG GGG ATG TTA AA (Pst1 -XmaI -GGS - STOP- BamHI)

Th4: CTT CCT TCA ATG TGG TGG TGG TGA TGA TGG (link beween Th1 Th2)

Th5: GGG CTG CAG CCA TGG GAA TTC T (link between Th2 and Th3)

Th6: GAT CTT TAA CAT CCC CC (link between Th3 and pVL, -pg67)

C Synthesis of ScFv DNA fragment

VH and VL regions of M350:

Total RNA isolated from M350 hybridome have been used as a template for a reverse transcription using oligo (dT) as primers (Reverse Transcription IBI Fermentas). A PCR realized with those cDNAs and specific primers (mouse Ig-Prime-Kit, Novagen) have led to the selective amplification of VH and VL chains. These regions are then cloned in "blunt" in pST-Blue 1 plasmid and sequenced.

VH and VL regions of other hybridoines:

Total RNA isolated from selected hybridome was used as a template for a reverse transcription using oligo (dT) (Reverse Transcription IBI Fermentas). A PCR with specific primers (mouse Ig-Prime-Kit, Novagen) led to the selective amplification of VH and VL chains. These products are then cloned in pGEMT (TA cloning System front PROMEGA) vector and sequenced. Three new VH and VL sequences were determined from clone therap.99B3 (**Figure 3**), clone therap.88E10 (**Figure 4**), and therap.152C3 (**Figure 5**).

Obtention of the ScFv-transfer vector:

VH-link-VL chimeric DNA were done by fusion-PCR in two steps (**Figure 12**). The first

step added a link-sequence (Gly-Gly-Gly-Gly-Ser) at the 3' of the VH chain and at the 5' end of the VL chain respectively. The second step was a PCR fusion leading to the chimeric DNA: VH-link-VL. The set of primers used in this second step brings a 5' -EcoRI and a 3'-XmaI sites to VH and VL respectively that will be used for the subcloning of the final product in pVL-PSgp671 vector (**Figure 13**).

D Cotransfection and purification of recombinant baculoviruses

Sf9 cells were cotransfected with viral DNA (BaculoGold ; Pharmingen) and recombinant transfer vector DNA (pVL-PSgp671-ScFv) by the lipofection method (Feloner and Ringold, 1989) (DOTAP; Roche). Screening and purification of recombinant viruses were carried out by the common procedure described by Summers and Smith (Summers and Smith, 1987). The recombinant virus was named BAC-PSgp671-scFv and amplified to constitute a viral stock with an M0I of 10^8.

E Analysis of recombinant proteins

Infected cells were collected, washed with cold phosphate-buffered saline (PBS) and resuspended in sample reducing buffer (Laemmli, 1970). After boiling (100°C for 5 min), proteins samples were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli, 1970). The apparent molecular weight of the protein was check by coomassie blue staining or the proteins were transferred onto a nitrocellulose filter (Schleicher and Schuell ; BAS 85, 0.45µm) with a semidry blotter apparatus (Ancos). The nitrocellulose membrane was then stained with Ponceau Red (Sigma) and subsequently blocked with a solution of' Tris-saline buffer (0.05 M Tris-HCI ph7.4, 0.2 M NaC1) containing 0.05% Tween 20 and 5% non fat milk (TS-sat). ScFv was detected using a mouse monoclonal antibody raised against His(6)-Tag (SIGMA) as primary antibody and a sheep anti-mouse immunoglobulin G (IgG)- horseradish peroxydase conjugate as secondary antibody (1; 3000 Amersham). The immunoreactive bands were visualized by using ECL reagents as described by the manufacturer (Amersham).

F Protein production and purification

To obtain viral stock, Sf9 insect cells cultured in IPL41 medium and 5% FCS are infected in exponential phase with the recombinant baculoviruses at MOI1. After a 7-day incubation period at 28° in IPL41 medium with 5% FCS, the supernatant is harvested by centrifugation at 8000 RPM during 15 min. Then High-five insect cells cultured in Xpress media (Biowhitaker) are infected with recombinant baculovirus in exponential phase at MOI 10, following lh30 of infection High Five cells were harvested by centrifugation and resuspended in Xpress media without serum. After a 4-day period of incubation at 28°C, the supenatant is harvested by centrifugation at 8000 RPM during 15 min. These supernatants are then concentrated by two rounds of ammonium sulfate precipitation. The precipitate obtained by sedimentation is dialyzed during 12 hours and purified using batch of Ni-NTA agarose beads as described by the manufacturer (Qiagen). After dialysis (2 days, PBS, 4°C) and analysis by Coomassic staining purified proteins were used for the covalent association with cytotoxic peptides.

EXAMPLE 3

Method of coupling ScFv to pTox

The peptide was assembled using Fmoc solid phase peptide synthesis, after the last Fmoc deprotection a propionyloxy succinimide ester was allowed to react, in the presence of diisopropyl ethylamine, with the alpha amino group of the peptide. At the end of the reaction (30 min) the peptide resin was washed with methylene chloride and the peptide was classically cleaved and deprotected under acidic conditions. The activated peptide was then purified by HPLC and its integrity was confirmed by mass spectrometry. The activated peptide was then allowed to react with the ScFv with peptide in a molar ratio of 10:1 (pH7, PBS, glass tube over agitation for 3 hours at room temperature). Then, dialysis was done for 48h against PBS a 4°C. Four Tox peptides were coupled to ScFv using this method:

Tox 11	ScFv-M350-Jac5 (Vpr71-96[C761])
Ctr1ToX11I	ScFv-M350-Jac5M (Vpr71-96[C76S;R73,80A])
Tox 12	ScFv-Vpr52-96[C76S]
Ctr1Tox12	ScFv -Vpr52-96[C76S ; R73A; R80A]

EXAMPLE 4

Examples of Targ-Tox or Targ-Save structures

All the Tox peptides can have a facultative N-terminal biotin and a facultative C-terminal amide fonction. Tox0 is a Tox peptide which does not necessarily require an association with a Targ. Tox1, Tox2, Tox 5, Tox6, Save1, Save2 and their respective control can posses a facultative gly-gly- (-GG-) linker between the Targ and the Tox/Save motif.

Tox0	Biot-DTWTGVEALIRILQQLLFIHFRIGCRHSRIGIIQQRRTRNGASKS
Ctr1Tox0	Biot-DTWTGVEALIRILQQLLFHFAIGCRHSAIGIIQQRRTRNGASKS

Tox1	Biot- CNGRC-GG-HFRIGCRHSRIG
Ctr1Tox1	Biot- CNGRC-GG-HFAIGCRHSAIG
Ctr2Tox1	Biot-CNGRC-GG-CNGRC
Ctr3Tox1	Biot-GG-HFRIGCRHSRIG
Ctr4Tox1	Biot-CNGRC-GG-Scramble
Ctr5Tox1	Biot-KETWWETWWTEW-GG-HFRIGCRHSRIG

Tox2	Biot-ACDCRGDCFC-GG-HFRIGCRHSRIG
Ctr1Tox2	Biot- ACDCRGDCFC-GG-HFAIGCRHSAIG

Tox5

Tox5	Biot-CNGRC-GG-DKRTQFWRYFPGN (hANT2m)
Ctr1Tox5	Biot-CNGRC-GG-DKRTQFWRYFAGN (hANT2)
Ctr2Tox5	Biot-CNGRC-GG-DRHKQFWRYFPGN (hANT1m)
Ctr3Tox5	Biot-CNGRC-GG-DKHTQFWRYFPGN (hANT3m)
Ctr4Tox5	Biot-GG-DKRTQFWRYFPGN (hANT2m)
Ctr5Tox5	Biot-GG-DRHKQFWRYFPGN (hANT1m)
Ctr6Tox5	Biot-GG-DKHTQFWRYFPGN (hANT3m)
Ctr7Tox5	Biot-CNGRC-GG-Scramble

Tox6

Tox6	Biot-ACDCRGDCFC-GG-DKRTQFWRYFPGN (hANT2m)
Ctr1Tox6	Biot-ACDCRGDCFC-GG-DKRTQFWRYFAGN (hANT2)
Ctr2Tox6	Biot-ACDCRGDCFC-GG-DRHKQFWRYFPGN (hANT1m)
Ctr3Tox6	Biot-ACDCRGDCFC-GG-DKHTQFWRYFPGN (hANT3m)
Ctr4Tox6	Biot-ACDCRGDCFC-GG
Ctr5Tox6	Biot-ACDCRGDCFC-GG-Scramble

Tox 11

Tox 11	ScFv-M350-Jac5(Vpr71-96[C76])
Ctr1Tox11	ScFv-M350-Jac5M(Vpr71-96[C76;R73,80A])

Save1

Save1	Biot-RKKRRQRRR-DKRTQFWRYFAGN (hANT2)
Ctr1Save1	Biot-RKKRRQRRR-DKRTQFWRYFPGN (hANT2m)
Ctr2Save1	Biot-RKKRRQRRR-DRHKQFWRYFAGN (hANT1)
Ctr3Save1	Biot-RKKRRQRRR-DKHTQFWRYFAGN (hANT3)
Ctr4Save1	Biot-RKKRRQRRR
Ctr5Save1	Biot-RKKRRQRRR-Scramble

Save2

Save 2	Biot-RKKRRQRRR-LASGGAAGATSLCFVYPL (hANT[117-134])
Ctr1Save2	Biot-RKKRRQRRR-GAWSNVLRGMGGAFVLVLY (ANTTM6[271-289])
Ctr2Save2	Biot-RKKRRQRRR-scramble

EXAMPLE 5

Evaluation of mitochondrial and nuclear parameters of Apoptosis in cells (cell lines) and cell-free systems

A. Cells

MCF-7, MDA-MB231, COS and HeLa cells are cultured in complete culture medium (DMEM supplemented with 2 mM glutamine, 10% FCS, 1 mM Pyruvate, 10 mM Hepes and 100 U/ml pencillin/streptomycin). Jurkat cells expressing CD4 and stably transfected with the human Bcl-2 gene or a Neomycin (Neo) resistance vector [Aillet, *et al.*, 1998 J. Virol. 72:9698-9705] only were kindly provided by N. Israel (Pasteur Institute, Paris). Neo and Bcl-2 U937 cells [Zamzami *et al.*, 1995 J. Exp. Med], and CEM-C7 cells are cultured in RPMI 1640 Glutamax medium supplemented with 10% FCS, antibiotics, and 0.8 μg/ml G418.

The cell **tests** that have been implemented determine the pathway (intracellular penetration, then subcellular localization) of the candidates, and the apoptotic status ($\Delta\psi m$, activation and relocalization of cell death effectors, content in nuclear DNA) of the target cell. In order to determine these parameters it is necessary to use fluorescent probes to label the cells and/or the candidates molecules and to implement the following two analytical procedures : multi-parameter cytofluorimetry and fluorescent microscopy. As far as neuroprotection is

concerned, tests were carried out on primary cultures of cortical neuronal cells from mice embryos. As far as cardioprotection is concerned, tests were carried out on primary cultures of cardiomyocytes from mice embryos.

- **Intra-cellular pathway tests:**. the TARG-TOX ou TARG-SAVE peptides coupled either with biotin (detected using fluorochromes conjugated with streptavidin ; or by ligand-blot after subcellular fractioning) or with FITC (detected by direct observation of living cells, videomicroscopy and image analysis) are added to the cells. It possible to favor the TOX or SAVE mitochondrial routing by inserting mitochondrial addressing signals (the Apoptosis Inducing Factor or ornithin transcarbamylase, for example). Similarly, the mitochondrial routing is evaluated after modifying sequences and certain lateral chains (phosphorylations, methylations), then replacing the peptides by peptidomimetics.

- **Multi-parameter analysis of apoptosis** on tumoral and endothelial cell lines, and primary neurons. Fluorescents probes wil be used to mesure the state of the mitochondrial transmembrane potential (JCI, DioC6, mitoTrackers) and nuclear condensation (Hoescht). Similarly, the post-mitochondrial parameters of apoptosis are evaluated using classical hypoploidy tests and cell surface labeling with annexin V-FITC.

In this type of tests, we evaluate either the cytotoxic potential of the TARG-TOX, i.e. their capacity to kill (via a mitochondrial effect) tumoral ou endothelial cell lines (the best TARG-TOX must also kill over-expressing Bel-2 cell lines); or the cytoprotective potential of the TARG-SAVE when the neurons are subjected to different apoptogenic treatments.

B. Apoptosis Modulation

PBS-washed cells (1-5 x 10^5 /ml) are incubated with (1 to 5 µM) of pTarg-pTox in complete culture medium supplemented or not with cyclosporin A (CsA; 1 µM), bongkrekic acid (BA; 50 µM), and/or the caspase inhibitors N-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk; 50 µM; Bachem Bioscience, Inc.), Boc-Asp-fluoromethylketone (Boc-D.fmk), or *N*-benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA.fmk; all used at 100 µM added each 24 h; Enzyme Systems). During exposure to pTarg-pTox, human primary PBLs from healthy donors, purified with Lymphoprep (Pharmacia), are cultured in RPMI 1640 Glutamax medium without any addition of serum. In contrast, PHA blasts (24 h of 1 µg/ml PHA-P [Wellcome Industries]; 48 h with 100 U/ml human recombinant IL-2 [Boehringer Mannheim]) are cultured with 10% FCS.

C. Cytofluorimetric Determinations of Apoptosis-associated Alterations in Intact Cells

For cytofluorometry, the following fluorochromes are employed: 3,3'-dihexyloxacarbocyanine iodide (DiOC(6)3; 40 nM) for mitochondrial transmembrane potential ($\Delta\Psi m$) quantification, hydroethidine (4 µM) for the determination of superoxide anion generation, and propidium. iodide (PI; 5 µM) for the determination of viability (Zamzami, N., *et al.*, 1995. J. Exp. Med. 182:367-377). The frequency of subdiploid cells is determined by PI (50 µg/ml) staining of ethanol-permeabilized cells treated with 500 µg/ml RNase (Sigma Chemical Co.; 30 min, room temperature [RT]) in PBS, pH 7.4, supplemented with 5 mM glucose (Nicoletti, I. *et al.*, 1991. J. Immunol. Methods. 139:271-280).

D. Fluorescence staining of life cells and immunofluorescence

For the assessment of mitochondrial and nuclear features of apoptosis, cells cultured on a cover slip are incubated with the $\Delta\Psi m$-sensitive dyes chloromethyl-X-rosamine (CMXRos; 50 nM; Molecular Probes, Inc.) or 5,5',6,6'-tetrachloro-l,l', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 2 µM, Molecular Probes), the $\Delta\Psi m$-insensitive dye Mitotracker green (1 µM; Molecular Probes, Inc.), and/or Hoechst 33342 (2 µM, Sigma) for 30 min at 37°C in complete culture medium (.Marzo, I*et al.* 1998. Science. 281:2027-2031).

E. For in situ determinations of pTarg-pTox internalisation

For *in situ* determinations of TARG-(MLS)-TOX/SAVE internalisation, cells are incubated at different times with TARG-(MLS)-TOX/SAVE, and then cells are fixed with 4% paraformaldehyde and 0.19% picric acid in PBS (pH 7.4) for 1 h at RT. Fixed cells are permeabilized with 0.1% SDS in PBS at RT (for 5 min), blocked with 10% FCS, and stained with an mAb specific for hexa-histidine tag (clone HIS-1, IgG2a, SIGMA) revealed by a goat anti-mouse PE conjugate [Southern Biotechnology Associates, Inc.]), Hsp60 (mAb H4149 [Sigma Chemical Co.], revealed by a goat anti-mouse IgGl FITC conjugate), cytochrome c oxidase (COX; mAb 20E8-C12 [Molecular Probes, Inc.], revealed by a goat anti-mouse IgG2a FITC conjugate), or when the Targ is a biotinylated peptide, a streptavidin-PE reagent is added 30 min. followed by detection of the fluorescence intensity by fluorescence (and/or confocal) microscopy.

F. Assessment of mitochondrial parameters in vitro

Mitochondria are purified from rat liver, as described (Costantini *et al.*, 1996), and resuspended in 250 mM sucrose + 0.1 mM EGTA + 10 mM –tris[hydroxymethyl]methyl-2-Aminoethanesulfonic acid, pH=7.4). For the induction of PT, mitochondria (0.5 mg protein per ml) are resuspended in PT buffer (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 5 mM Tris-succinate, 1 mM Tris-phosphate, 2 µM rotenone, and 10 µM EGTA-Tris), and monitored in an F4500 fluorescence spectrometer (Hitachi, Tokyo, Japan) for the 90° light scattering of light (545 nm) to determine large amplitude swelling after addition of 2 mM atractyloside (Atr), 1 µM cyclosporin A (CsA; Novartis, Basel, Switzerland), 5 µM $CaCl_2$, and/or 0.5 to 20 µM of pTarg-pTox or pTarg-pSave. For the determination of the $\Delta\Psi_m$, mitochondria (0.5 mg protein per ml) are incubated in a buffer supplemented with 1 µM rhodamine 123 (Molecular Probes, Eugene, OR) and the dequenching of rhodamine fluorescence (excitation 505 nm, emission 525 nm) is measured as described (Shimizu *et al.*, 1998). Supernatants from mitochondria (6800 g for 15min; then 20 000 g for 1 h; 4°C) are frozen at -80°C until determination of apoptogenic activity on isolated nuclei, DEVD-afc cleaving activity, and immunodetection of cytochrome c and *AIF*. Cytochrome *c* and *AIF* are detected by means of a monoclonal antibody (clone 7H8.2C12, Pharmingen) and a polyclonal rabbit anti-serum (Susin *et al.* 1999) respectively.

Swelling of isolated mitochondria

Table F1 :

Tox0, Tox1, Tox5, Tox6 induce permeability transition pore (PTP) openning

Name of molecules 5 μM	Induction of Mitochondrial swelling (sw) +++ rapid sw ; ++ low sw ; + very low sw ; - no sw t 20 min
-	-
Tox0	+++
Tox1	++
Ctr1Tox1	-
Ctr2Tox1	-
Ctr3Tox1	+
Ctr4Tox1	-
Tox5	++
Tox6	++

Table F2:
Save 1 and Save2 inhibit atractyloside-induced PTP openning

Name of molecules	Mitrochondrial swelling (sw) %
-	2
Ca 2+ 100μM	100
Atractyloside 600μM	110
Save I 5μM	2
Save I 5μM + Atr 600μM	5
Save I 20μM	12
Save I 20μM + Atr 600μM	12
Save II 10μM	2
Save II 20μM	16
Save II 10μM + Atr 600μM	16
Save II 20μM + Atr 600μM	16

G. ANT purification and reconstitution in liposomes

ANT was purified from rat heart mitochondria as previously described (8). After mechanical shearing, mitochondria were suspended in 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 200 µM EDTA, 100 mM DTT, 0.5 mg/ml subtilisin, pH7.4, kept 8 min on ice and sedimented twice by differential centrifugations (5 min, 500 x g, and 10 min, 10,000 x g). Mitochondrial proteins were solubilized by 6% [v:v] Triton X-100 (Boehringer Mannheim) in 40 mM K_2HPO_4, 40 mM KC1, 2 mM EDTA, pH 6.0, for 6 min at RT and solubilized proteins were recovered by ultracentrifugation (30 min, 24,000 x g, 4°C). Then, 2 ml of this Triton X-100 extract was applied to a column filled with 1 g of hydroxyapatite (BioGel HTP, BioRad), eluted with previous buffer and diluted [v:v] with 20 mM MES, 200 µM EDTA, 0.5% Triton X-100, pH6.0. Subsequently, the sample was separated with a Hitrap SP column using a FPLC system (Pharmacia) and a linear NaCl gradient (0-1M). Proteins concentration was determined using microBCA-assay (Pierce, Rockfoll, Illinois). Purified ANT and/or recombinant Bcl-2 were reconstituted in PC/cardiolipin liposomes. Briefly, to prepare liposomes, 45 mg PC and 1 mg cardiolipin were mixed in 1 ml chloroform, and the solvent was evaporated under nitrogen. Dry lipids were resuspended in 1 ml liposome buffer (125 mM sucrose + 10 mM -2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid; Hepes, pH 7.4) containing 0.3% n-octyl-β-D-pyranoside and mixed by continuous vortexing for 40 min at RT. ANT (0.1 mg/ml) or recombinant Bcl-2 (0.1 mg/ml) were then mixed with liposomes [v:v] and incubated for 20 min at RT. Proteoliposomes were finally dialysed overnight at 4°C.

H. Pore opening assay

ANT-proteoliposomes are sonicated in the presence of 1 mM 4-MUP and 10 mM KC1 (50W, 22sec, Branson sonifier 250) on ice as previously described (28). Then, liposomes were separated on Sepadex G-25 columns (PD-10, Pharmacia) from unencapsulated products. 25 µl-aliquots of liposomes were diluted to 3 ml in 10 mM Hepes, 125 mM saccharose, pH 7.4, mixed with various concentrations of the proapoptotic inducers and incubated for 1 h at RT. Potential inhibitors of mitochondrial membranes permeabilization such as BA, ATP and ADP, were added to the liposomes 30 min before treatment. After addition of 10 µl-alkaline phosphatase (5 U/ml, Boehringer Mannheim) diluted in liposomes buffer + 0.5 mM $MgCl_2$, samples were incubated

for 15 min at 37°C under agitation and the enzymatic conversion of 4-MUP in 4-MU was stopped by addition of 150 µl Stop buffer (10 mM Hepes-NaOH, 200 mM EDTA, pH 10). The 4-MU-dependent fluorescence (360/450 nm) was subsequently quantitated (28) using a Perkin Elmer spectrofluorimeter. Atractyloside, a pro-apoptotic permeability transition inducer, was used in each experiment as a standard to determine the 100% response. The percentage of 4-MUP release induced by Vpr-derived peptides or pTarg-ptox was calculated as following : [(fluorescence of liposomes treated by pTar-pTox - fluorescence of untreated liposomes) / (fluorescence of liposomes treated by atractyloside - fluorescence of untreated liposomes)] x 100.

ANT pore opening assay:

Table H1 : examples of fuctionnal interaction between ANT and Tox or Save constructs.

Tox0 and Tox6 induce ANT-protéoliposomes permeabilisation. Save1 and Save2 block Atractyloside (Atra) -induced ANT-protéoliposomes permeabilisation

molecules	Permeabilisation of ANT - proteoliposomes +++ high UMP release ; ++ UMP release ; + low UMP release ; - no UMP release
-	-
Atra 50µM	+
Atra 100µM	++
Atra 200µM	+++
Tox0 (Biotin-Vpr52-96) 2µM	+++
Tox6 5µM	++
Biotin-Vpr71-96[C76S] 5µM	++
Save1 5µM	-
Atra 200µM + Save1 5µM	-
Save2 5µM	-
Atra 200µM + Save2 5µM	-

I. Binding assays and western blot

Mouse liver mitochondria were isolated as described (zamzami *et al.*, 2000). For the determination of cytochrome C release, supernatants from pTarg-pTox treated mitochondria (6800 g for 15min; then 20 000 g for 1 h; 4°C) were frozen at -80°C until immunodetection of cytochrome c (mouse monoclonal antibody clone 7H8.2CI2, Pharmingen). For binding assays, purified mitochondria were incubated (250 µg of protein in 100 µl swelling buffer) for 30 min at

RT 5 μM (binding assay) of pTarg-pTox or biotin-pTarg-pTox. Mitochondria were lysed either after incubation with biotinylated Vpr52-96 (upper panel) or lysed before (lower panel) with 150 μl of a buffer containing 20 mM Tris/HCl, pH 7.6; 400 mM NaCl, 50 mM KCl, 1mM EDTA, 0.2 mM PMSF, aprotinin (100U/ml), 1% Triton X-100 and 20% glycerol. Such extracts were diluted with 2 volumes of PBS plus lmM EDTA before the addition of 150 μl avidin-agarose (ImmunoPure, from Pierce) to capture the biotin-labeled Vpr52-96 complexed with its mitochondrial ligand(s) (2 hours at 4 °C in a roller drum). The avidin-agarose was washed batchwise with PBS (5 x 5 ml; 1000 g, 5 min, 4 °C), resuspended in 100 μl of 2 fold concentrated Laemmli buffer containing 4% SDS and 5 mM β-mercaptoethanol, incubated 10 min at RT and centrifuged (1000 g, 10 min, 4 °C). Finally, the supernatants were heated at 95 °C for 5 min and analysed by SDS-PAGE (12%), followed by Western blot and immunodetection with a rabbit polyclonal anti-serum against human ANT (kindly provided by Dr. Heide H. Schmid; The Hormel Institute, University of Minnesota, MI; Ref).

J. Flow cytometric analysis of purified mitochondria

Mouse liver mitochondria are isolated as described (zamzami *et al.*, 2000). Purified mitochondria are resuspended in PT buffer (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 5 mM Tris-succinate, 1 mM Tris-phosphate, 2 μM rotenone, and 10 μM EGTA). Cytofluorometric (FACSVantage, Beckton Dickinson) detection is restricted to mitochondria by gating on the FSC/SSC parameters and on the main peak of the FSC-W parameter. Confirmation *a posteriori* of the validity of these double gating is obtained by labeling of mitochondria with the $\Delta\Psi_m$-insensitive mitochondrial dye MitoTracker® Green (75 nM; Molecular Probes; green fluorescence). To determine the percentage of mitochondria having a low $\Delta\Psi_m$, the $\Delta\Psi_m$-sensitive fluorochrome JC-1 (200 nM; 570-595 nm) is added 10 min before CCCP or pTarg-pTox molecules. Percentage of mitochondria having a low$\Delta\Psi_m$, is determined in dot-plot FSC/FL-2 (red fluorescence) windows.

K. Cell-free system of apoptosis

AIF activity in the supernatant of mitochondria is tested on HeLa cell nuclei, as described (Susin *et al.*, 1997b). Briefly, AIF-containing supernatants of mitochondria are added to purified HeLa nuclei (90 min, 37 °C), which are stained with propidium iodide (PI; 10 μg/ml; Sigma Chemical Co.) and analyzed in an Elite II cytofluorometer (Coulter) to determine the frequency

of hypoploid nuclei. In some experiments isolated mitochondria, cytosols from Jurkat or CEM cells (prepared as described (Susin *et al.,* 1997a)), and/or pTarg-pTox are added to the nuclei. Caspase activity in the mitochondrial supernatant was measured using Ac-DEVD-amido-4-trifluoromethylcoumarin (Bachem Bioscience, Inc.) as fluorogenic substrate.

L. Purification and reconstitution of PTPC in liposomes

PTPC from Wistar rat brains are purified and reconstituted in liposomes following published protocols (Brenner *et al.*, 1998; Marzo *et al.*, 1998b). Briefly, homogenized brains are subjected to the extraction of triton-soluble proteins, adsorption of proteins to a DE52 resin anion exchange column, elution on a KC1 gradient, and incorporation of fractions with maximum hexokinase activity into phosphatidyl choline/cholesterol (5: 1, w:w) vesicles by overnight dialysis. Recombinant human Bcl-2 (1-218) lacking the hydrophobic transmembrane domain (Δ219-239), produced and purified as described (Schendel *et al.*, 1997) are added during the dialysis step at a dose corresponding to 5% of the total PTPC proteins (approximately 10 ng Bcl-2 per mg lipids). Liposomes recovered from dialysis are ultrasonicated. (120 W) during 7 sec in 5 mM malate and 10 mM KCl, charged on a Sephadex G50 columns (Pharmacia), and eluted with 125 mM sucrose + 10 mM HEPES (pH 7.4). Aliquots (approx. 10^7) of liposomes are incubated during 60 min at RT in 125 mM sucrose + 10 mM HEPES (pH 7.4) in the presence or absence of pTarg-pTox, [52-96]Vpr or atractyloside. Then, liposomes are equilibrated with 3,3'dihexylocarbocyanine iodide ($DiOC_6(3)$, 80 nM, 20-30 min at RT; Molecular Probes), and analyzed in a FACS-Vantage cytofluorometer (Becton Dickinson, San José, CA, USA) for $DiOC_6(3)$ retention, as described (Brenner *et al.*, 1998; Marzo *et al.*, 1998b).

Triplicates of 5×10^4 liposomes are analyzed and results are expressed as % of reduction of $DiOC_6(3)$ fluorescence, considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Examples of specific peptides and constructs relating to this invention that can be utilized in carrying out the foregoing techniques are shown in Tables I, II, and III, as well as any chimeric molecule that is a combination between TARG and TOX or TARG and SAVE peptides or peptidomimetics.

EXAMPLE 6

Surface plasmon resonance indicates that Tox0, Tox1, Tox5, Tox6, Save1 binds purified ANT but not purified VDAC.

Methodology.

Sensor Chips SA (streptavidin coated sensor chips) were used for immobilisation of the different peptides. Tox1 was immobilised at a density of 0.7 ng/mm^2, Tox0 at a density of 3.7 ng/mm^2,. Ctr1Tox0 at a density of 1.4 ng/mm^2 , Tox5 at a density of 1 ng/mm^2 , Tox6 at a density of 1 ng/mm2 , Save1 at a density of 1.3 ng/mm^2 , and the control peptide at a density of 0.8 ng/mm^2 . Association and dissociation kinetics of ANT and VDAC interactions were followed at a rate of 10 μL/min for 10 minutes (5 minutes association and 5 minutes dissociation). The ligand was regenerated with a 1 minute flux of KSCN 3M. The obtained sensorgrams were analysed by the BlAeval 3.1 software using the method of double references (Myszka D.G. 2000. Kinetic, equilibrium and thermodynamic analysis of macromolecular interactions with BIACORE. *Methods Enzymol.* 323:325-340). From the sensorgrams with the ligands were first substracted the sensorgrams obtained with the corresponding analyte solvents. A second substraction was performed with the sensorgrams obtained with the control peptide ligand. The control peptide for the Tox and Save peptides was biot-H19C corresponding to the sequence of the β2-adrenergic receptor (Lebesgue D., Wallukat G., Mijares A., Granier C., Argibay J., and Hoebeke J. (1998) An agonist-like monoclonal antibody to the human β 2-adrenergic receptor. *Eur.J. Pharmacol.* 348:123-133). The control peptide for Tox0 was Ctr1Tox0.

Results.

Figure 6 shows the interaction between ANT and Vpr for 4 ANT concentrations (6.25 to 50 nM). The sensorgrams were best analysed using the simple Lagmuir model with drifting baseline and resulted in a Kd of 0.15 nM with a Rmax of 160 ($x2 = 7.24$). The same analysis was performed for the sensorgrams showing the interaction between ANT and Tox1 (**Figure 7**). Studying the VDAC interaction both with Tox0 and Tox1 at VDAC concentrations which were ten times higher (**Figure 8** and **9**), the sensorgrams showed only extremely low association with

the peptide ligand and the obtained curves could not be analysed by the different Langmuir bindings models.

Thee other peptides were tested for their interaction with ANT at a concentration of 50 nM (**Figure 10**). Purified ANT recognised Tox5, Tox6, and Save1 with relative affinities of respectively 0.1, 0.7, and 0.01 nM. These value being obtained at only one ANT concentration only give the relative affinity of ANT for the three peptides. Again, the use of 50 nM VDAC to interact with the same peptides did not result in any specific binding as shown in **Figure 11**.

The following references have been cited herein. The entire disclosure of each reference cited herein is relied upon and incorporated by reference herein.

1-D. Decaudin, I. Marzo, C. Brenner and G. Kroemer. Mitochondria in chemotherapy-induced apoptosis: A prospective novel target of cancer therapy. Int J Oncol, *12*: 141-52, 1998.
2-S. Fulda, S. A. Susin, G. Kroemer and K. M. Debatin. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. Cancer Res, *58*: 4453-60, 1998a.
3-S. Fulda, C. Scaffidi, S. A. Susin, P. H. Krammer, G. Kroemer, M. E. Peter and K. M. 4-Debatin. Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. J Biol Chem, *273*: 33942-8, 1998b.
4-L. Ravagnan, I. Marzo, P. Costantini, S. A. Susin, N. Zamzami, P. X. Petit, F. Hirsch, M. Goulbern, M. F. Poupon, L. Miccoli, Z. Xie, J. C. Reed and G. Kroemer. Lonidamine triggers apoptosis via a direct, Bcl-2-inhibited effect on the mitochondrial permeability transition pore. Oncogene, *18*: 2537-46, 1999.
5-N. Larochette, D. Decaudin, E. Jacotot, C. Brenner, I. Marzo, S. A. Susin, N. Zamzami, Z. Xie, J. Reed and G. Kroemer. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res, *249*: 413-21, 1999.
6-P. Marchetti, N. Zamzami, B. Joseph, S. Schraen-Maschke, C. Mereau-Richard, P. Costantini, D. Metivier, S. A. Susin, G. Kroemer and P. Formstecher. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. Cancer Res, *59*: 6257-66, 1999.

7-P. Costantini, E. Jacotot, D. Decaudin and G. Kroemer. Mitochondrion as a novel target of anticancer chemotherapy. J. Natl. Cancer Inst., *92*: 1042-1053, 2000.

8-1. Marzo, C. Brenner, N. Zamzami, S. A. Susin, G. Beutner, D. Brdiczka, R. Remy, Z. H. Xie, J. C. Reed and G. Kroemer. The permeability transition pore complex: a target for apoptosis regulation by caspases and Bcl-2-related proteins. J Exp Med, *187*: 1261-71, 1998a.

9-I. Marzo, C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. A. Vieira, M. C. Prévost, Z. Xie, S. Matsuyama, J. C. Reed and G. Kroemer. Bax and Adenine Nucleotide Translocator Cooperate in the Mitochondrial Control of Apoptosis. Science, *281*: 2027-2031, 1998b.

10-Y. Tsujimoto, L. R. Finger, J. Yunis, P. C. Nowell and C. M. Croce. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science, *226*: 1097-9, 1984.

11-J. C. Reed, M. Cuddy, T. Slabiak, C. M. Croce and P. C. Nowell. Oncogenic potential of Bcl-2 demonstrated by gene transfer. Nature, *336*: 259-61, 1988.

12-M. Narita, S. Shimizu, T. Ito, T. Chittenden, R. J. Lutz, H. Matsuda and Y. Tsujimoto. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A, *95*: 14681-6, 1998.

13-C. Brenner, H. Cadiou, H. L. Vieira, N. Zamzami, 1. Marzo, Z. Xie, B. Leber, D. Andrews, H. Duclohier, J. C. Reed and G. Kroemer. Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator. Oncogene, *19*: 329-36, 2000.

14-E. Jacotot, L. Ravagnan, M. Loeffler, K. F. Ferri, H. L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J. P. Briand, T. lrinopoulou, E. Daugas, S. A. Susin, D. Cointe, Z. H. Xie, J. C. Reed, B. P. Roques and G. Kroemer. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J Exp Med, *191*: 33-46, 2000.

15-P. Costantini, A. S. Belzacq, H. L. Vieira, N. Larochette, M. A. de Pablo, N. Zamzami, S. A. Susin, C. Brenner and G. Kroemer. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene, *19*: 307-14, 2000.

16-S. Shimizu, A. Konishi, T. Kodama and Y. Tsujimoto. BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc Natl Acad Sci U S A, *97:* 3100-5, 2000.

17-M. G. VanderHeiden, N. S. Chandel, P. T. Schumacker and C. B. Thompson. Bcl-$x_{(L)}$ prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Molecular Cell, 3: 159-167, 1999.

18-Griffioen, A.W., Molema, G. (2000). Angiogenesis: Potentials for Pharmacologic Intervention in the Treatment of Cancer, Cardiovascular Diseases, and Chronic Inflammation. Pharmacological Reviews 52:237-268.

What is claimed is:

1. Method for inducing or preventing the apoptosis of.eukaryotic cells comprising the homing on specific tissue cell population of a chimeric bifunctional molecule able to modulate-the activity of permeability transition pore complex (PTPC).
2. A method according to claim 1, wherein said chimeric molecules modulate the activity of the permeability transition pore complex (PTPC) of a specific eukaryotic cell by the regulation of opening or the closing of said pore.
3. A method according to claim 1 or 2, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target specifically a tissue cell population, and the second functional molecule has the function to regulate the apoptosis activity linked to the permeability transition pore complex (PTPC) of said specific calls.
4. A method according to claim 3, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target and to enter specifically in a tissue cell population and the second functional molecule has the function to regulate the apoptosis activity linked to the permeability transition pore complex (PTPC) of said specific cells.
5. A method according to claim 3, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target and to enter specifically in a tissue cell population of interest and the second functional molecule has the function to target specifically and inducing or preventing the death of said cells by apoptosis by the regulation of the

opening or the closing of the permeability transition pore complex (PTPC) of mitochondria or a fragment thereof.

6. A method according to claim 4, wherein said chimeric molecule has the formula:

Targ-Tox,

wherein Tox is a viral or a retroviral apoptotic peptide or a peptidomimetic or a fragment of a protein that interacts with permeability transition pore complex (PTPC) of a specific eukaryotic cell to cause apoptosis of the cell; and Targ is chosen from:

an antibody,

an antibody fragment,

arecombinant antibody fragment,

M350/ScFv,

V461/ScFv,

a homing peptide, and

any peptide chosen in Table III,

wherein said molecule binds and enters the cell specifically.

7. A method according to claim 5, wherein said chimeric molecule has the formula

Targ-Save,

wherein Save is a viral or a retroviral or a cellular antiapoptotic peptide or peptidomimetic or a fragment of protein that interacts with permeability transition pore complex (PTPC) of a specific eukaryotic cell to prevent the apoptosis of the cell with the proviso that when Save peptide is a viral peptide, Save is not vMIA protein of Cytomegalovirus; and

Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

a homing peptide, and

any peptide chosen in Table III,

wherein said molecule binds and enters the cell specifically.

8. A method according to anyone of claims 1 to 7, wherein said chimeric molecules comprises a Mitochondrial Localisation Sequence (MLS), which has the function to address specifically the second functional molecule to mitochondrial or intermembrane space-of the mitochondria.

9. A method according to claims 1, 2, 3, 4, 5, 6 and 8, wherein Tox is chosen from the group of peptides of Table I.

10. A method according to claims 1, 2, 3, 4, 5, and 7, wherein Save is chosen from the group of peptides of Table II.

11. A method according to any one of claims 1 to 10, wherein the second functional molecule of said chimeric molecules has the function to interact specifically with ANT of the PTPC of mitochondria also refers to as adenine nucleotide translocator isoforms 1, 2, or 3.

12. A chimeric bifunctional molecule capable to enter specifically in a tissue cell population for induce or prevent death of said cell by apoptosis and comprising at least a first functional molecule covalently linked to a second functional molecule, wherein said first

functional molecule has the function to target and to enter specifically in a tissue cell population of interest and the second functional molecule has the function to target specifically and inducing or preventing the death of said cells by apoptosis by the regulation of the opening or the closing of the permeability transition pore complex (PTPC) of mitochondria or a fragment thereof.

13. A chimeric molecule according to claim 12 which has the formula:

Targ-Tox,

wherein Tox is a viral or a retroviral apoptotic peptide or peptidomimetic or a fragment of a protein that interacts with PermeabilityTransifion Pore Complex (PTPC) of a specific eukaryofic cell to cause apoptosis of the cell; and

Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

V461/ScFv,

a homing peptide, and

any peptide of Table III,

wherein said molecule binds and enters the cell specifically.

14. A chimeric molecule according to claim 12 which has of the formula

Targ-Save

Wherein Save is a viral or a retroviral or a cellular antiapoptotic peptide or peptidomimetic or a fragment of protein that interacts with Permeability Transition. Pore Complex (PTPC) of a specific eukaryotic cell to prevent apoptosis of the cell, with the proviso that when Save peptide is a viral peptide, Save is not vMIA protein of Cytomegalovirus;

and Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

a homing peptide, and

any peptide of Table III,

wherein said molecule binds and enters the cell specifically.

15. A chimeric molecule according to any of claims 12 to 14 comprising a mitochondrial localisation sequence (MLS) which has the function to address specifically the second functional molecule to mitochondrial membranes or intermembrane space.

16. A chimeric molecule according to claims 13 or 15, wherein Tox is chosen from the group of peptides of Table I.

17. A chimeric molecule according to claims 14 and 15, wherein wherein Save is chosen from the group of peptides of Table II.

18. A chimeric molecule according to claims 13, 15 and 16, wherein the Targ and Tox peptides are covalently bonded through a peptide linker comprising 3 to 18 amino acids.

19. A chimeric molecule according to claims 14, 15 and 17, wherein the Targ and Save peptides are covalently bonded through a peptide linker comprising 3 to 18 amino acids.

20. A vector encoding a chimeric molecule as claimed in any one of claims 12 to 19.

21. A hybridoma secreting Targ according to claim 13 or 14 and deposited at the National Collection of Culture and Microorganism (C.N.C.M.) on January 24, 2001, under the accession number n° I 2617.

22. A purified monoclonal antibody encoded by the hybridoma of claim 21.

23. A recombinant host cell comprising a vector as claimed in claim 20.

24. A cancer cell having a tumor associated antigen on the surface thereof to which is bound the chimeric molecule as claimed in any one of claims 12 to 19.

25. A method of determining the presence of a cancer cell having a tumor-associated antigen on the surface thereof in a biological sample comprising :

    a) contacting a biological sample of interest with a chimeric peptide molecule according to claims 12 to 19 under conditions to permit the binding between the chimeric peptide according to the invention and the antigen on the surface of the. cancer cell,

    b) detecting the binding by usual technique; and

    c) optionally quantifying the binding detected in step b).

26. A method for inducing death by apoptosis in a tumoral or viral infected cell having a tumor-associated antigen on surface thereof in a biological sample comprising: contacting a biological sample of interest with a chimeric peptide molecule according to claims 16 or 17 under conditions to permit the binding between the chimeric peptide

according to the invention and the antigen on the surface of the cancer cell and for a time sufficient to allow the entry inside the cell and death cell by apoptosis or viral infected cells.

27. A method for prevent cell death by mitochondrial apoptosis comprising contacting a biological sample of interest with a chimeric molecule, **molecule** according to claims 17 or 19 under conditions to permit the binding between the chimeric molecule according to the invention and the cell of interest and for a time sufficient to allow the entry inside cell of interest and prevent the cell death by apoptosis.

28. A method for prevent cell death according to claim 27, wherein the cells of interest are choosen among the following cell populations: neurons, cardiocytes, and hepatocytes.

29. A method for identifying an active agent of interest that interacts with the activity of the permeability transition pore complex (PTPC) comprising

   a) contacting a biological sample containing cells with permeability transition pore complex (PTPC) with a chimeric peptide according to claims 12 to 19 in the presence of a candidate agent; and

   b) comparing the binding of the chimeric peptide with the permeability transition pore complex (PTPC) in absence of said agent.

   c) optionally, testing the activity of said selected agent on a preparation of a cellular extract comprising subcellular elements with the permeability transition pore complex (PTPC).

30. A method for identifying an active agent of interest that interacts with ANT peptide of permeability transition pore complex (PTPC) comprising:

d) contacting a biological sample containing cells with ANT peptide of permeability transition pore complex (PTPC) with a chimeric peptide according to claims 12 to 19 in the presence of a candidate agent; and

e) comparing the binding of the chimeric peptide with the ANT peptide of the permeability transition pore complex (PTPC) in absence of said agent.

f) optionally, testing the activity of said selected agent on a preparation of a cellular extract comprising subcellular elements with the ANT peptide of the permeability transition pore complex (PTPC).

31. A method of identification of mitochondrial antigen, said antigen having the capacity to interact with a macromolecule or a molecule or a peptide carrying the characteristic of Tox according to claims 13 or 16.

32. A method of identification of mitochondrial antigen, said antigen having the capacity to interact with a macromolecule or a molecule or a peptide carrying the characteristic of save according to claims 14 or 17.

33. A method of treatment or of prevention of a pathological infection or disease comprising the administration to a patient of the pharmaceutical composition containing at least a chimeric molecule according to any of claims 12 to 19.

34. A pharmaceutical composition comprising at least a chimeric molecule according to claims any of 12 to 19.

DNA Strider™ 1.2 ### Mardi 16 janvier 2001 22:01:48

pACgp67-ScFv461 -> 1-phase Translation

Fig. 1A

DNA sequence 10517 b.p. AAGCTTTACTCG ... ACGGCCAGTGCC linear

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1/1                                     31/11                                   61/21
AAG CTT TAC TCG TAA AGC GAG TTG AAG GAT CAT ATT TAG TTG CGT TTA TGA GAT AAG ATT GAA AGC ACG TGT AAA ATG TTT CCC GCG CGT
lys leu tyr ser OCH ser glu leu lys asp his ile AMB leu arg leu OPA asp lys ile glu ser thr cys lys met phe pro ala arg
91/31                                   121/41                                  151/51
TGG CAC AAC TAT TTA CAA TGC GGC CAA GTT ATA AAA GAT TCT AAT CTG ATA TGT TTT AAA ACA CCT TTG CGG CCC GAG TTG TTT GCG TAC
trp his asn tyr leu gln cys gly gln val ile lys asp ser asn leu ile cys phe lys thr pro leu arg pro glu leu phe ala tyr
181/61                                  211/71                                  241/81
GTG ACT AGC GAA GAA GAT GTG TGG ACC GCA GAA CAG ATA GTA AAA CAA AAC CCT AGT ATT GGA GCA ATA ATC GAT TTA ACC AAC ACG TCT
val thr ser glu glu asp val trp thr ala glu gln ile val lys gln asn pro ser ile gly ala ile ile asp leu thr asn thr ser
271/91                                  301/101                                 331/111
AAA TAT TAT GAT GGT GTG CAT TTT TTG CGG GCG GGC CTG TTA TAC AAA AAA ATT CAA GTA CCT GGC CAG ACT TTG CCG CCT GAA AGC ATA
lys tyr tyr asp gly val his phe leu arg ala gly leu leu tyr lys lys ile gln val pro gly gln thr leu pro pro glu ser ile
361/121                                 391/131                                 421/141
GTT CAA GAA TTT ATT GAC ACG GTA AAA GAA TTT ACA GAA AAG TGT CCC GGC ATG TTG GTG GGC GTG CAC TGC ACA CAC GGT ATT AAT CGC
val gln glu phe ile asp thr val lys glu phe thr glu lys cys pro gly met leu val gly val his cys thr his gly ile asn arg
451/151                                 481/161                                 511/171
ACC GGT TAC ATG GTG TGC AGA TAT TTA ATG CAC ACC CTG GGT ATT GCG CCG CAG GAA GCC ATA GAT AGA TTC GAA AAA GCC AGA GGT CAC
thr gly tyr met val cys arg tyr leu met his thr leu gly ile ala pro gln glu ala ile asp arg phe glu lys ala arg gly his
541/181                                 571/191                                 601/201
AAA ATT GAA AGA CAA AAT TAC GTT CAA GAT TTA TTA ATT TAA TTA ATA TTA TTT GCA TTC TTT AAC AAA TAC TTT ATC CTA TTT TCA AAT
lys ile glu arg gln asn tyr val gln asp leu leu ile OCH leu ile leu phe ala phe phe asn lys tyr phe ile leu phe ser asn
631/211                                 661/221                                 691/231
TGT TGC GCT TCT TCC AGC GAA CCA AAA CTA TGC TTC GCT TGC TCC GTT TAG CTT GTA GCC GAT CAG TGG CGT TGT TCC AAT CGA CGG TAG
cys cys ala ser ser ser glu pro lys leu cys phe ala cys ser val AMB leu val ala asp gln trp arg cys ser asn arg arg AMB
721/241                                 751/251                                 781/261
GAT TAG GCC GGA TAT TCT CCA CCA CAA TGT TGG CAA CGT TGA TGT TAC GTT TAT GCT TTT GGT TTT CCA CGT ACG TCT TTT GGC CGG TAA
asp AMB ala gly tyr ser pro pro gln cys trp gln arg OPA cys tyr val tyr ala phe gly phe pro arg thr ser phe gly arg OCH
811/271                                 841/281                                 871/291
TAG CCG TAA ACG TAG TGC CGT CGC GCG TCA CGC ACA ACA CCG GAT GTT TGC GCT TGT CCG CGG GGT ATT GAA CCG CGC GAT CCG ACA AAT
AMB pro OCH thr AMB cys arg arg ala ser arg thr thr pro asp val cys ala cys pro arg gly ile glu pro arg asp pro thr asn
901/301                                 931/311                                 961/321
CCA CCA CTT TGG CAA CTA AAT CGG TGA CCT GCG CGT CTT TTT TCT GCA TTA TTT CGT CTT TCT TTT GCA TGG TTT CCT GGA AGC CGG TGT
pro pro leu trp gln leu asn arg OPA pro ala arg leu phe ser ala leu phe arg leu ser phe ala trp phe pro gly ser arg cys
991/331                                 1021/341                                1051/351
ACA TGC GGT TTA GAT CAG TCA TGA CGC GCG TGA CCT GCA AAT CTT TGG CCT CGA TCT GCT TGT CCT TGA TGG CAA CGA TGC GTT CAA TAA
thr cys gly leu asp gln ser OPA arg ala OPA pro ala asn leu trp pro arg ser ala cys pro OPA trp gln arg cys val gln OCH
1081/361                                1111/371                                1141/381
ACT CTT GTT TTT TAA CAA GTT CCT CGG TTT TTT GCG CCA CCA CCG CTT GCA GCG CGT TTG TGT GCT CGG TGA ATG TCG CAA TCA GCT TAG
thr leu val phe OCH gln val pro arg phe phe ala pro pro pro leu ala ala arg leu cys ala arg OPA met ser gln ser ala AMB
1171/391                                1201/401                                1231/411
TCA CCA ACT GTT TGC TCT CCT CCT CCC GTT GTT TGA TCG CGG GAT CGT ACT TGC CGG TGC AGA GCA CTT GAG GAA TTA CTT CTT CTA AAA
ser pro thr val cys ser pro pro pro val val OPA ser arg asp arg thr cys arg cys arg ala leu glu glu leu leu leu leu lys
1261/421                                1291/431                                1321/441
GCC ATT CTT GTA ATT CTA TGG CGT AAG GCA ATT TGG ACT TCA TAA TCA GCT GAA TCA CGC CGG ATT TAG TAA TGA GCA CTG TAT GCG GCT
ala ile leu val ile leu trp arg lys ala ile trp thr ser OCH ser ala glu ser arg arg ile AMB OCH OPA ala leu tyr ala ala
1351/451                                1381/461                                1411/471
GCA AAT ACA GCG GGT CGC CCT TTT TCA CGA CGC TGT TAG AGG TAG GGC CCC CAT TTT GGA TGG TCT GCT CAA ATA ACG ATT TGT ATT TAT
ala asn thr ala gly arg pro phe ser arg arg cys AMB arg AMB gly pro his phe gly trp ser ala gln ile thr ile cys ile tyr
1441/481                                1471/491                                1501/501
TGT CTA CAT GAA CAC GTA TAG CTT TAT CAC AAA CTG TAT ATT TTA AAC TGT TAG CGA CGT CCT TGG CCA CGA ACC GGA CCT GTT GGT CGC
cys leu his glu his val AMB leu tyr his lys leu tyr ile leu asn cys AMB arg arg pro trp pro arg thr gly pro val gly arg
1531/511                                1561/521                                1591/531
GCT CTA GCA CGT ACC GCA GGT TGA ACG TAT CTT CTC CAA ATT TAA ATT CTC CAA TTT TAA CGC GAG CCA TTT TGA TAC ACG TGT GTC GAT
ala leu ala arg thr ala gly OPA thr tyr leu leu gln ile OCH ile leu gln phe OCH arg glu pro phe OPA tyr thr cys val asp
1621/541                                1651/551                                1681/561
TTT GCA ACA ACT ATT GTT TTT TAA CGC AAA CTA AAC TTA TTG TGG TAA GCA ATA ATT AAA TAT GGG GGA ACA TGC GCC GCT ACA ACA CTC
phe ala thr thr ile val phe OCH arg lys leu asn leu leu trp OCH ala ile ile lys tyr gly gly thr cys ala ala thr thr leu
1711/571                                1741/581                                1771/591
GTC GTT ATG AAC GCA GAC GGC GCC GGT CTC GGC GCA AGC GGC TAA AAC GTG TTG CGC GTT CAA CGC GGC AAA CAT CGC AAA AGC CAA TAG
val val met asn ala asp gly ala gly leu gly ala ser gly OCH asn val leu arg val gln arg gly lys his arg lys ser gln AMB
1801/601                                1831/611                                1861/621
TAC AGT TTT GAT TTG CAT ATT AAC GGC GAT TTT TTA AAT TAT CTT ATT TAA TAA ATA GTT ATG ACG CCT ACA ACT CCC CGC CCG CGT TGA
tyr ser phe asp leu his ile asn gly asp phe leu asn tyr leu ile OCH OCH ile val met thr pro thr thr pro arg pro arg OPA
1891/631                                1921/641                                1951/651
CTC GCT GCA CCT CGA GCA GTT CGT TGA CGC CTT CCT CCG TGT GGC CGA ACA CGT CGA GCG GGT GGT CGA TGA CCA GCG GCG TGC CGC ACG
leu ala ala pro arg ala val arg OPA arg leu pro pro cys gly arg thr arg arg ala gly gly arg OPA pro ala ala cys arg thr
1981/661                                2011/671                                2041/681
CGA CGC ACA AGT ATC TGT ACA CCG AAT GAT CGT CGG GCG AAG GCA CGT CGG CCT CCA AGT GGC AAT ATT GGC AAA TTC GAA AAT ATA TAC
arg arg thr ser ile cys thr pro asn asp arg arg ala lys ala arg arg pro pro ser gly asn ile gly lys phe glu asn ile tyr
2071/691                                2101/701                                2131/711
AGT TGG GTT GTT TGC GCA TAT CTA TCG TGG CGT TGG GCA TGT ACG TCC GAA CGT TGA TTT GCA TGC AAG CCG AAA TTA AAT CAT TGC GAT
ser trp val val cys ala tyr leu ser trp arg trp ala cys thr ser glu arg OPA phe ala cys lys pro lys leu asn his cys asp
2161/721                                2191/731                                2221/741
TAG TGC GAT TAA AAC GTT GTA CAT CCT CGC TTT TAA TCA TGC CGT CGA TTA AAT CGC GCA ATC GAG TCA AGT GAT CAA AGT GTG GAA TAA
AMB cys asp OCH asn val val his pro arg phe OCH ser cys arg arg leu asn arg ala ile glu ser ser asp gln ser val glu OCH
2251/751                                2281/761                                2311/771
TGT TTT CTT TGT ATT CCC GAG TCA AGC GCA GCG CGT ATT TTA ACA AAC TAG CCA TCT TGT AAG TTA GTT TCA TTT AAT GCA ACT TTA TCC
cys phe leu cys ile pro glu ser ser ala ala arg ile leu thr asn AMB pro ser cys lys leu val ser phe asn ala thr leu ser
2341/781                                2371/791                                2401/801
AAT AAT ATA TTA TGT ATC GCA CGT CAA GAA TTA ACA ATG CGC CCG TTG TCG CAT CTC AAC ACG ACT ATG ATA GAG ATC AAA TAA AGC GCG
asn asn ile leu cys ile ala arg gln glu leu thr met arg pro leu ser his leu asn thr thr met ile glu ile lys OCH ser ala
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pACgp67-ScFv461 -> 1-phase Translation

Fig 1B

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2431/811                                2461/821                                2491/831
AAT TAA ATA GCT TGC GAC GCA ACG TGC ACG ATC TGT GCA CGC GTT CCG GCA CGA GCT TTG ATT GTA ATA AGT TTT TAC GAA GCG ATG ACA
asn OCH ile ala cys asp ala thr cys thr ile cys ala arg val pro ala arg ala leu ile val ile ser phe tyr glu ala met thr
2521/841                                2551/851                                2581/861
TGA CCC CCG TAG TGA CAA CGA TCA CGC CCA AAA GAA CTG CCG ACT ACA AAA TTA CCG AGT ATG TCG GTG ACG TTA AAA CTA TTA AGC CAC
OPA pro pro AMB OPA gln arg ser arg pro lys glu leu pro thr thr lys leu pro ser met ser val thr leu lys leu leu ser his
2611/871                                2641/881                                2671/891
CCA ATC GAC CGT TAG TCG AAT CAG GAC CGC TGG TGC GAG AAG CCG CGA AGT ATG GCG AAT GCA TCG TAT AAC GTG TGG AGT CCG CTC ATT
pro ile asp arg AMB ser asn gln asp arg trp cys glu lys pro arg ser met ala asn ala ser tyr asn val trp ser pro leu ile
2701/901                                2731/911                                2761/921
AGA GCG TCA TGT TTA GAC AAG AAA GCT ACA TAT TTA ATT GAT CCC GAT GAT TTT ATT GAT AAA TTG ACC CTA ACT CCA TAC ACG GTA TTC
arg ala ser cys leu asp lys lys ala thr tyr leu ile asp pro asp asp phe ile asp lys leu thr leu thr pro tyr thr val phe
2791/931                                2821/941                                2851/951
TAC AAT GGC GGG GTT TTG GTC AAA ATT TCC GGA CTG CGA TTG TAC ATG CTG TTA ACG GCT CCG CCC ACT ATT AAT GAA ATT AAA AAT TCC
tyr asn gly gly val leu val lys ile ser gly leu arg leu tyr met leu leu thr ala pro pro thr ile asn glu ile lys asn ser
2881/961                                2911/971                                2941/981
AAT TTT AAA AAA CGC AGC AAG AGA AAC ATT TGT ATG AAA GAA TGC GTA GAA GGA AAG AAA AAT GTC GTC GAC ATG CTG AAC AAC AAG ATT
asn phe lys lys arg ser lys arg asn ile cys met lys glu cys val glu gly lys lys asn val val asp met leu asn asn lys ile
2971/991                                3001/1001                               3031/1011
AAT ATG CCT CCG TGT ATA AAA AAA ATA TTG AAC GAT TTG AAA GAA AAC AAT GTA CCG CGC GGC GGT ATG TAC AGG AAG AGG TTT ATA CTA
asn met pro pro cys ile lys lys ile leu asn asp leu lys glu asn asn val pro arg gly gly met tyr arg lys arg phe ile leu
3061/1021                               3091/1031                               3121/1041
AAC TGT TAC ATT GCA AAC GTG GTT TCG TGT GCC AAG TGT GAA AAC CGA TGT TTA ATC AAG GCT CTG ACG CAT TTC TAC AAC CAC GAC TCC
asn cys tyr ile ala asn val val ser cys ala lys cys glu asn arg cys leu ile lys ala leu thr his phe tyr asn his asp ser
3151/1051                               3181/1061                               3211/1071
AAG TGT GTG GGT GAA GTC ATG CAT CTT TTA ATC AAA TCC CAA GAT GTG TAT AAA CCA CCA AAC TGC CAA AAA ATG AAA ACT GTC GAC AAG
lys cys val gly glu val met his leu leu ile lys ser gln asp val tyr lys pro pro asn cys gln lys met lys thr val asp lys
3241/1081                               3271/1091                               3301/1101
CTC TGT CCG TTT GCT GGC AAC TGC AAG GGT CTC AAT CCT ATT TGT AAT TAT TGA ATA ATA AAA CAA TTA TAA ATG CTA AAT TTG TTT TTT
leu cys pro phe ala gly asn cys lys gly leu asn pro ile cys asn tyr OPA ile ile lys gln leu OCH met leu asn leu phe phe
3331/1111                               3361/1121                               3391/1131
ATT AAC GAT ACA AAC CAA ACG CAA CAA GAA CAT TTG TAG TAT TAT CTA TAA TTG AAA ACG CGT AGT TAT AAT CGC TGA GGT AAT ATT TAA
ile asn asp thr asn gln thr gln gln glu his leu AMB tyr tyr leu OCH leu lys thr arg ser tyr asn arg OPA gly asn ile OCH
3421/1141                               3451/1151                               3481/1161
AAT CAT TTT CAA ATG ATT CAC AGT TAA TTT GCG ACA ATA TAA TTT TAT TTT CAC ATA AAC TAG ACG CCT TGT CGT CTT CTT CTT CGT ATT
asn his phe gln met ile his ser OCH phe ala thr ile OCH phe tyr phe his ile asn AMB thr pro cys arg leu leu leu arg ile
3511/1171                               3541/1181                               3571/1191
CCT TCT CTT TTT CAT TTT TCT CCT CAT AAA AAT TAA CAT AGT TAT TAT CGT ATC CAT ATA TGT ATC TAT CGT ATA GAG TAA ATT TTT TGT
pro ser leu phe his phe ser pro his lys asn OCH his ser tyr tyr arg ile his ile cys ile tyr arg ile glu OCH ile phe cys
3601/1201                               3631/1211                               3661/1221
TGT CAT AAA TAT ATA TGT CTT TTT TAA TGG GGT GTA TAG TAC CGC TGC GCA TAG TTT TTC TGT AAT TTA CAA CAG TGC TAT TTT CTG GTA
cys his lys tyr ile cys leu phe OCH trp gly val AMB tyr arg cys ala AMB phe phe cys asn leu gln gln cys tyr phe leu val
3691/1231                               3721/1241                               3751/1251
GTT CTT CGG AGT GTG TTG CTT TAA TTA TTA AAT TTA TAT AAC CAA TGA ATT TGG GAT CGT CGG TTT TGT ACA ATA TGT TGC CGG CAT AGT
val leu arg ser val leu leu OCH leu leu asn leu tyr asn gln OPA ile trp asp arg arg phe cys thr ile cys cys arg his ser
3781/1261                               3811/1271                               3841/1281
ACG CAG CTT CTT CTA GTT CAA TTA CAC CAT TTT TTA GCA GCA CCG GAT TAA CAT AAC TTT CCA AAA TGT TGT ACG AAC CGT TAA ACA AAA
thr gln leu leu leu val gln leu his his phe leu ala ala pro asp OCH his asn phe pro lys cys cys thr asn arg OCH thr lys
3871/1291                               3901/1301                               3931/1311
ACA GTT CAC CTC CCT TTT CTA TAC TAT TGT CTG CGA GCA GTT GTT TGT TGT TAA AAA TAA CAG CCA TTG TAA TGA GAC GCA CAA ACT AAT
thr val his leu pro phe leu tyr tyr cys leu arg ala val val cys cys OCH lys OCH gln pro leu OCH OPA asp ala gln thr asn
3961/1321                               3991/1331                               4021/1341
ATC ACA AAC TGG AAA TGT CTA TCA ATA TAT AGT TGC TGA TAT CAT GGA GAT AAT TAA AAT GAT AAC CAT CTC GCA AAT AAA TAA GTA TTT
ile thr asn trp lys cys leu ser ile tyr ser cys OPA tyr his gly asp asn OCH asn asp asn his leu ala asn lys OCH val phe
4051/1351                               4081/1361                               4111/1371                    -> PS gp67
TAC TGT TTT CGT AAC AGT TTT GTA ATA AAA AAA CCT ATA AAT ATT CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CGC GGA TCT ATG CTA
tyr cys phe arg asn ser phe val ile lys lys pro ile asn ile pro asp tyr ser tyr arg pro thr ile gly arg gly ser met leu
4141/1381                               4171/1391                               4201/1401
CTA GTA AAT CAG TCA CAC CAA GGC TTC AAT AAG GAA CAC ACA AGC AAG ATG GTA AGC GCT ATT GTT TTA TAT GTG CTT TTG GCG GCG GCG
leu val asn gln ser his gln gly phe asn lys glu his thr ser lys met val ser ala ile val leu tyr val leu leu ala ala ala
4231/1411                               4261/1421    Tag HIS      Facteur Xa    4291/1431  -> ScFv461 = VH
GCG CAT TCT GCC TTT GCG GCG GAT CTT gga tcc CAT CAT CAC CAC CAC CAC atc gaa gga aga GAA TTC CAG GTG CAG CTG AAG GAG TCA
ala his ser ala phe ala ala asp leu gly ser his his his his his his ile glu gly arg glu phe gln val gln leu lys glu ser
4321/1441                               4351/1451                               4381/1461
GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC ACA TGC ACT GTC TCA GGG TTC TCA TTA ACC AGC TAT GGT GTA AGC TGG GTT
gly pro gly leu val ala pro ser gln ser leu ser ile thr cys thr val ser gly phe ser leu thr ser tyr gly val ser trp val
4411/1471                               4441/1481                               4471/1491
CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG GGA GTA ATA TGG GGT GAC GGG AGC ACA AAT TAT CAT TCA GCT CTC ATA TCC AGA CTG
arg gln pro pro gly lys gly leu glu trp leu gly val ile trp gly asp gly ser thr asn tyr his ser ala leu ile ser arg leu
4501/1501                               4531/1511                               4561/1521
AGC ATC AGC AAG GAT AAC TCC AAG AGC CAA GTT TTC TTA AAA CTG AAC AGT CTG CAA ACT GAT GAC ACA GCC ACG TAC TAC TGT GCC AAA
ser ile ser lys asp asn ser lys ser gln val phe leu lys leu asn ser leu gln thr asp asp thr ala thr tyr tyr cys ala lys
4591/1531                               4621/1541                               4651/1551                                 peptide
AGG GGA GGC TAT GGT AAC TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA   jond
arg gly gly tyr gly asn tyr tyr ala met asp tyr trp gly gln gly thr ser val thr val ser ser gly gly gly gly ser gly gly
4681/1561                      -> | VL  4711/1571                               4741/1581
GGT GGC TCT GGC GGT GGC GGA TCG GAC ATT GTG ATG ACC CAG TCT CAC AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG GTC AGC ATC ACC
gly gly ser gly gly gly gly ser asp ile val met thr gln ser his lys phe met ser thr ser val gly asp arg val ser ile thr
4771/1591                               4801/1601                               4831/1611
TGC AAG GCC AGT CAG GAT GTG AGT ACT GCT GTA GCC TGG TAT CAA CAA AAA CCA GGG CAA TCT CCT AAA CTA CTG ATT TAC TGG GCA TCC
cys lys ala ser gln asp val ser thr ala val ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp ala ser
4861/1621                               4891/1631                               4921/1641
ACC CGG CAC ACT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TAT ACT CTC ACC ATC AGC AGT GTG CAG GCT GAA GAC
thr arg his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp tyr thr leu thr ile ser ser val gln ala glu asp
4951/1651                               4981/1661                               5011/1671
CTG GCA CTT TAT TAC TGT CAG CAA CAT TAT AGC ACT CCT CCG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT CCC GGG gga
leu ala leu tyr tyr cys gln gln his tyr ser thr pro pro thr phe gly gly gly thr lys leu glu ile lys arg ala pro gly gly
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! CDR des régions variable et VL

fin ScFv   2 gly

pACgp67-ScFv461 -> 1-phase Translation   Fig. 1C   16/01/1 22:01:48 Page 3

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5041/1681 STOP                       5071/1691                                 5101/1701
tgt TAA aga tct gat ccT TTC CTG GGA CCC GGC AAG AAC CAA AAA CTC ACT CTC TTC AAG GAA ATC CGT AAT GTT AAA CCC GAC ACG ATG
cys OCH arg ser asp pro phe leu gly pro gly lys asn gln lys leu thr leu phe lys glu ile arg asn val lys pro asp thr met
5131/1711                            5161/1721                                 5191/1731
AAG CTT GTC GTT GGA TGG AAA GGA AAA GAG TTC TAC AGG GAA ACT TGG ACC CGC TTC ATG GAA GAC AGC TTC CCC ATT GTT AAC GAC CAA
lys leu val val gly trp lys gly lys glu phe tyr arg glu thr trp thr arg phe met glu asp ser phe pro ile val asn asp gln
5221/1741                            5251/1751                                 5281/1761
GAA GTG ATG GAT GTT TTC CTT GTT GTC AAC ATG CGT CCC ACT AGA CCC AAC CGT TGT TAC AAA TTC CTG GCC CAA CAC GCT CTG CGT TGC
glu val met asp val phe leu val val asn met arg pro thr arg pro asn arg cys tyr lys phe leu ala gln his ala leu arg cys
5311/1771                            5341/1781                                 5371/1791
GAC CCC GAC TAT GTA CCT CAT GAC GTG ATT AGG ATC GTC GAG CCT TCA TGG GTG GGC AGC AAC AAC GAG TAC CGC ATC AGC CTG GCT AAG
asp pro asp tyr val pro his asp val ile arg ile val glu pro ser trp val gly ser asn asn glu tyr arg ile ser leu ala lys
5401/1801                            5431/1811                                 5461/1821
AAG GGC GGC GGC TGC CCA ATA ATG AAC CTT CAC TCT GAG TAC ACC AAC TCG TTC GAA CAG TTC ATC GAT CGT GTC ATC TGG GAG AAC TTC
lys gly gly gly cys pro ile met asn leu his ser glu tyr thr asn ser phe glu gln phe ile asp arg val ile trp glu asn phe
5491/1831                            5521/1841                                 5551/1851
TAC AAG CCC ATC GTT TAC ATC GGT ACC GAC TCT GCT GAA GAG GAG GAA ATT CTC CTT GAA GTT TCC CTG GTG TTC AAA GTA AAG GAG TTT
tyr lys pro ile val tyr ile gly thr asp ser ala glu glu glu glu ile leu leu glu val ser leu val phe lys val lys glu phe
5581/1861                            5611/1871                                 5641/1881
GCA CCA GAC GCA CCT CTG TTC ACT GGT CCG GCG TAT TAA AAC ACG ATA CAT TGT TAT TAG TAC ATT TAT TAA GCG CTA GAT TCT GTG CGT
ala pro asp ala pro leu phe thr gly pro ala tyr OCH asn thr ile his cys tyr AMB tyr ile tyr OCH ala leu asp ser val arg
5671/1891                            5701/1901                                 5731/1911
TGT TGA TTT ACA GAC AAT TGT TGT ACG TAT TTT AAT AAT TCA TTA AAT TTA TAA TCT TTA GGG TGG TAT GTT AGA GCG AAA ATC AAA TGA
cys OPA phe thr asp asn cys cys thr tyr phe asn asn ser leu asn leu OCH ser leu gly trp tyr val arg ala lys ile lys OPA
5761/1921                            5791/1931                                 5821/1941
TTT TCA GCG TCT TTA TAT CTG AAT TTA AAT ATT AAA TCC TCA ATA GAT TTG TAA AAT AGG TTT CGA TTA GTT TCA AAC AAG GGT TGT TTT
phe ser ala ser leu tyr leu asn leu asn ile lys ser ser ile asp leu OCH asn arg phe arg leu val ser asn lys gly cys phe
5851/1951                            5881/1961                                 5911/1971
TCC GAA CCG ATG GCT GGA CTA TCT AAT GGA TTT TCG CTC AAC GCC ACA AAA CTT GCC AAA TCT TGT AGC AGC AAT CTA GCT TTG TCG ATA
ser glu pro met ala gly leu ser asn gly phe ser leu asn ala thr lys leu ala lys ser cys ser ser asn leu ala leu ser ile
5941/1981                            5971/1991                                 6001/2001
TTC GTT TGT GTT TTG TTT TGT AAT AAA GGT TCG ACG TCG TTC AAA ATA TTA TGC GCT TTT GTA TTT CTT TCA TCA CTG TCG TTA GTG TAC
phe val cys val leu phe cys asn lys gly ser thr ser phe lys ile leu cys ala phe val phe leu ser ser leu ser leu val tyr
6031/2011                            6061/2021                                 6091/2031
AAT TGA CTC GAC GTA AAC ACG TTA AAT AAA GCT TGG ACA TAT TTA ACA TCG GGC GTG TTA GCT TTA TTA GGC CGA TTA TCG TCG TCG TCC
asn OPA leu asp val asn thr leu asn lys ala trp thr tyr leu thr ser gly val leu ala leu leu gly arg leu ser ser ser ser
6121/2041                            6151/2051                                 6181/2061
CAA CCC TCG TCG TTA GAA GTT GCT TCC GAA GAC GAT TTT GCC ATA GCC ACA CGA CGC CTA TTA ATT GTG TCG GCT AAC ACG TCC GCG ATC
gln pro ser ser leu glu val ala ser glu asp asp phe ala ile ala thr arg arg leu leu ile val ser ala asn thr ser ala ile
6211/2071                            6241/2081                                 6271/2091
AAA TTT GTA GTT GAG CTT TTT GGA ATT ATT TCT GAT TGC GGG CGT TTT TGG GCG GGT TTC AAT CTA ACT GTG CCC GAT TTT AAT TCA GAC
lys phe val val glu leu phe gly ile ile ser asp cys gly arg phe trp ala gly phe asn leu thr val pro asp phe asn ser asp
6301/2101                            6331/2111                                 6361/2121
AAC ACG TTA GAA AGC GAT GGT GCA GGC GGT GGT AAC ATT TCA GAC GGC AAA TCT ACT AAT GGC GGC GGT GGT GGA GCT GAT GAT AAA TCT
asn thr leu glu ser asp gly ala gly gly gly asn ile ser asp gly lys ser thr asn gly gly gly gly gly ala asp asp lys ser
6391/2131                            6421/2141                                 6451/2151
ACC ATC GGT GGA GGC GCA GGC GGG GCT GGC GGC GGA GGC GGA GGC GGA GGT GGT GGC GGT GAT GCA GAC GGC GGT TTA GGC TCA AAT GTC
thr ile gly gly gly ala gly gly ala gly gly gly gly gly gly gly gly gly gly gly asp ala asp gly gly leu gly ser asn val
6481/2161                            6511/2171                                 6541/2181
TCT TTA GGC AAC ACA GTC GGC ACC TCA ACT ATT GTA CTG GTT TCG GGC GCC GTT TTT GGT TTG ACC GGT CTG AGA CGA GTG CGA TTT TTT
ser leu gly asn thr val gly thr ser thr ile val leu val ser gly ala val phe gly leu thr gly leu arg arg val arg phe phe
6571/2191                            6601/2201                                 6631/2211
TCG TTT CTA ATA GCT TCC AAC AAT TGT TGT CTG TCG TCT AAA GGT GCA GCG GGT TGA GGT TCC GTC GGC ATT GGT GGA GCG GGC GGC AAT
ser phe leu ile ala ser asn asn cys cys leu ser ser lys gly ala ala gly OPA gly ser val gly ile gly gly ala gly gly asn
6661/2221                            6691/2231                                 6721/2241
TCA GAC ATC GAT GGT GGT GGT GGT GGT GGA GGC GCT GGA ATG TTA GGC ACG GGA GAA GGT GGT GGC GGC GGT GCC GCC GGT ATA ATT TGT
ser asp ile asp gly gly gly gly gly gly gly ala gly met leu gly thr gly glu gly gly gly gly gly ala ala gly ile ile cys
6751/2251                            6781/2261                                 6811/2271
TCT GGT TTA GTT TGT TCG CGC ACG ATT GTG GGC ACC GGC GCA GGC GCC GCT GGC TGC ACA ACG GAA GGT CGT CTG CTT CGA GGC AGC GCT
ser gly leu val cys ser arg thr ile val gly thr gly ala gly ala ala gly cys thr thr glu gly arg leu leu arg gly ser ala
6841/2281                            6871/2291                                 6901/2301
TGG GGT GGT GGC AAT TCA ATA TTA TAA TTG GAA TAC AAA TCG TAA AAA TCT GCT ATA AGC ATT GTA ATT TCG CTA TCG TTT ACC GTG CCG
trp gly gly gly asn ser ile leu OCH leu glu tyr lys ser OCH lys ser ala ile ser ile val ile ser leu ser phe thr val pro
6931/2311                            6961/2321                                 6991/2331
ATA TTT AAC AAC CGC TCA ATG TAA GCA ATT GTA TTG TAA AGA GAT TGT CTC AAG CTC CGC ACG CCG ATA ACA AGC CTT TTC ATT TTT ACT
ile phe asn asn arg ser met OCH ala ile val leu OCH arg asp cys leu lys leu arg thr pro ile thr ser leu phe ile phe thr
7021/2341                            7051/2351                                 7081/2361
ACA GCA TTG TAG TGG CGA GAC ACT TCG CTG TCG TCG ACG TAC ATG TAT GCT TTG TTG TCA AAA ACG TCG TTG GCA AGC TTT AAA ATA TTT
thr ala leu AMB trp arg asp thr ser leu ser ser thr tyr met tyr ala leu leu ser lys thr ser leu ala ser phe lys ile phe
7111/2371                            7141/2381                                 7171/2391
AAA AGA ACA TCT CTG TTC AGC ACC ACT GTG TTG TCG TAA ATG TTG TTT TTG ATA ATT TGC GCT TCC GCA GTA TCG ACA CGT TCA AAA AAT
lys arg thr ser leu phe ser thr thr val leu ser OCH met leu phe leu ile ile cys ala ser ala val ser thr arg ser lys asn
7201/2401                            7231/2411                                 7261/2421
TGA TGC GCA TCA ATT TTG TTG TTC CTA TTA TTG AAT AAA TAA GAT TGT ACA GAT TCA TAT CTA CGA TTC GTC ATG GCC ACC ACA AAT GCT
OPA cys ala ser ile leu leu phe leu leu leu asn lys OCH asp cys thr asp ser tyr leu arg phe val met ala thr thr asn ala
7291/2431                            7321/2441                                 7351/2451
ACG CTG CAA ACG CTG GTA CAA TTT TAC GAA AAC TGC AAA AAC GTC AAA ACT CGG TAT AAA ATA ATC AAC GGG CGC TTT GGC AAA ATA TCT
thr leu gln thr leu val gln phe tyr glu asn cys lys asn val lys thr arg tyr lys ile ile asn gly arg phe gly lys ile ser
7381/2461                            7411/2471                                 7441/2481
ATT TTA TCG CAC AAG CCC ACT AGC AAA TTG TAT TTG CAG AAA ACA ATT TCG GCG CAC AAT TTT AAC GCT GAC GAA ATA AAA GTT CAC CAG
ile leu ser his lys pro thr ser lys leu tyr leu gln lys thr ile ser ala his asn phe asn ala asp glu ile lys val his gln
7471/2491                            7501/2501                                 7531/2511
TTA ATG AGC GAC CAC CCA AAT TTT ATA AAA ATC TAT TTT AAT CAC GGT TCC ATC AAC AAC CAA GTG ATC GTG ATG GAC TAC ATT GAC TGT
leu met ser asp his pro asn phe ile lys ile tyr phe asn his gly ser ile asn asn gln val ile val met asp tyr ile asp cys
7561/2521                            7591/2531                                 7621/2541
CCC GAT TTA TTT GAA ACA CTA CAA ATT AAA GGC GAG CTT TCG TAC CAA CTT GTT AGC AAT ATT ATT AGA CAG CTG TGT GAA GCG CTC AAC
pro asp leu phe glu thr leu gln ile lys gly glu leu ser tyr gln leu val ser asn ile ile arg gln leu cys glu ala leu asn
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pACgp67-ScFv461 -> 1-phase Translation

Fig. 1D

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7651/2551                                7681/2561                                7711/2571
GAT TTG CAC AAG CAC AAT TTC ATA CAC AAC GAC ATA AAA CTC GAA AAT GTC TTA TAT TTC GAA GCA CTT GAT CGC GTG TAT GTT TGC GAT
asp leu his lys his asn phe ile his asn asp ile lys leu glu asn val leu tyr phe glu ala leu asp arg val tyr val cys asp
7741/2581                                7771/2591                                7801/2601
TAC GGA TTG TGC AAA CAC GAA AAC TCA CTT AGC GTG CAC GAC GGC ACG TTG GAG TAT TTT AGT CCG GAA AAA ATT CGA CAC ACA ACT ATG
tyr gly leu cys lys his glu asn ser leu ser val his asp gly thr leu glu tyr phe ser pro glu lys ile arg his thr thr met
7831/2611                                7861/2621                                7891/2631
CAC GTT TCG TTT GAC TGG TAC GCG GCG TGT TAA CAT ACA AGT TGC TAA CCG GCG GTT CGT AAT CAT GGT CAT AGC TGT TTC CTG TGT GAA
his val ser phe asp trp tyr ala ala cys OCH his thr ser cys OCH pro ala val arg asn his gly his ser cys phe leu cys glu
7921/2641                                7951/2651                                7981/2661
ATT GTT ATC CGC TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GCA TAA AGT GTA AAG CCT GGG GTG CCT AAT GAG TGA GCT AAC TCA CAT
ile val ile arg ser gln phe his thr thr tyr glu pro glu ala OCH ser val lys pro gly val pro asn glu OPA ala asn ser his
8011/2671                                8041/2681                                8071/2691
TAA TTG CGT TGC GCT CAC TGC CCG CTT TCC AGT CGG GAA ACC TGT CGT GCC AGC TGC ATT AAT GAA TCG GCC AAC GCG CGG GGA GAG GCG
OCH leu arg cys ala his cys pro leu ser ser arg glu thr cys arg ala ser cys ile asn glu ser ala asn ala arg gly glu ala
8101/2701                                8131/2711                                8161/2721
GTT TGC GTA TTG GGC GCT CTT CCG CTT CCT CGC TCA CTG ACT CGC TGC GCT CGG TCG TTC GGC TGC GGC GAG CGG TAT CAG CTC ACT CAA
val cys val leu gly ala leu pro leu pro arg ser leu thr arg cys ala arg ser phe gly cys gly glu arg tyr gln leu thr gln
8191/2731                                8221/2741                                8251/2751
AGG CGG TAA TAC GGT TAT CCA CAG AAT CAG GGG ATA ACG CAG GAA AGA ACA TGT GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA
arg arg OCH tyr gly tyr pro gln asn gln gly ile thr gln glu arg thr cys glu gln lys ala ser lys arg pro gly thr val lys
8281/2761                                8311/2771                                8341/2781
AGG CCG CGT TGC TGG CGT TTT TCC ATA GGC TCC GCC CCC CTG ACG AGC ATC ACA AAA ATC GAC GCT CAA GTC AGA GGT GGC GAA ACC CGA
arg pro arg cys trp arg phe ser ile gly ser ala pro leu thr ser ile thr lys ile asp ala gln val arg gly gly glu thr arg
8371/2791                                8401/2801                                8431/2811
CAG GAC TAT AAA GAT ACC AGG CGT TTC CCC CTG GAA GCT CCC TCG TGC GCT CTC CTG TTC CGA CCC TGC CGC TTA CCG GAT ACC TGT CCG
gln asp tyr lys asp thr arg arg phe pro leu glu ala pro ser cys ala leu leu phe arg pro cys arg leu pro asp thr cys pro
8461/2821                                8491/2831                                8521/2841
CCT TTC TCC CTT CGG GAA GCG TGG CGC TTT CTC ATA GCT CAC GCT GTA GGT ATC TCA GTT CGG TGT AGG TCG TTC GCT CCA AGC TGG GCT
pro phe ser leu arg glu ala trp arg phe leu ile ala his ala val gly ile ser val arg cys arg ser phe ala pro ser trp ala
8551/2851                                8581/2861                                8611/2871
GTG TGC ACG AAC CCC CCG TTC AGC CCG ACC GCT GCG CCT TAT CCG GTA ACT ATC GTC TTG AGT CCA ACC CGG TAA GAC ACG ACT TAT CGC
val cys thr asn pro pro phe ser pro thr ala ala pro tyr pro val thr ile val leu ser pro thr arg OCH asp thr thr tyr arg
8641/2881                                8671/2891                                8701/2901
CAC TGG CAG CAG CCA CTG GTA ACA GGA TTA GCA GAG CGA GGT ATG TAG GCG GTG CTA CAG AGT TCT TGA AGT GGT GGC CTA ACT ACG GCT
his trp gln gln pro leu val thr gly leu ala glu arg gly met AMB ala val leu gln ser ser OPA ser gly gly leu thr thr ala
8731/2911                                8761/2921                                8791/2931
ACA CTA GAA GGA CAG TAT TTG GTA TCT GCG CTC TGC TGA AGC CAG TTA CCT TCG GAA AAA GAG TTG GTA GCT CTT GAT CCG GCA AAC AAA
thr leu glu gly gln tyr leu val ser ala leu cys OPA ser gln leu pro ser glu lys glu leu val ala leu asp pro ala asn lys
8821/2941                                8851/2951                                8881/2961
CCA CCG CTG GTA GCG GTG GTT TTT TTG TTT GCA AGC AGC AGA TTA CGC GCA GAA AAA AAG GAT CTC AAG AAG ATC CTT TGA TCT TTT CTA
pro pro leu val ala val val phe leu phe ala ser ser arg leu arg ala glu lys lys asp leu lys lys ile leu OPA ser phe leu
8911/2971                                8941/2981                                8971/2991
CGG GGT CTG ACG CTC AGT GGA ACG AAA ACT CAC GTT AAG GGA TTT TGG TCA TGA GAT TAT CAA AAA GGA TCT TCA CCT AGA TCC TTT TAA
arg gly leu thr leu ser gly thr lys thr his val lys gly phe trp ser OPA asp tyr gln lys gly ser ser pro arg ser phe OCH
9001/3001                                9031/3011                                9061/3021
ATT AAA AAT GAA GTT TTA AAT CAA TCT AAA GTA TAT ATG AGT AAA CTT GGT CTG ACA GTT ACC AAT GCT TAA TCA GTG AGG CAC CTA TCT
ile lys asn glu val leu asn gln ser lys val tyr met ser lys leu gly leu thr val thr asn ala OCH ser val arg his leu ser
9091/3031                                9121/3041                                9151/3051
CAG CGA TCT GTC TAT TTC GTT CAT CCA TAG TTG CCT GAC TCC CCG TCG TGT AGA TAA CTA CGA TAC GGG AGG GCT TAC CAT CTG GCC CCA
gln arg ser val tyr phe val his pro AMB leu pro asp ser pro ser cys arg OCH leu arg tyr gly arg ala tyr his leu ala pro
9181/3061                                9211/3071                                9241/3081
GTG CTG CAA TGA TAC CGC GAG ACC CAC GCT CAC CGG CTC CAG ATT TAT CAG CAA TAA ACC AGC CAG CCG GAA GGG CCG AGC GCA GAA GTG
val leu gln OPA tyr arg glu thr his ala his arg leu gln ile tyr gln gln OCH thr ser gln pro glu gly pro ser ala glu val
9271/3091                                9301/3101                                9331/3111
GTC CTG CAA CTT TAT CCG CCT CCA TCC AGT CTA TTA ATT GTT GCC GGG AAG CTA GAG TAA GTA GTT CGC CAG TTA ATA GTT TGC GCA ACG
val leu gln leu tyr pro pro pro ser ser leu leu ile val ala gly lys leu glu OCH val val arg gln leu ile val cys ala thr
9361/3121                                9391/3131                                9421/3141
TTG TTG CCA TTG CTA CAG GCA TCG TGG TGT CAC GCT CGT CGT TTG GTA TGG CTT CAT TCA GCT CCG GTT CCC AAC GAT CAA GGC GAG TTA
leu leu pro leu leu gln ala ser trp cys his ala arg arg leu val trp leu his ser ala pro val pro asn asp gln gly glu leu
9451/3151                                9481/3161                                9511/3171
CAT GAT CCC CCA TGT TGT GCA AAA AAG CGG TTA GCT CCT TCG GTC CTC CGA TCG TTG TCA GAA GTA AGT TGG CCG CAG TGT TAT CAC TCA
his asp pro pro cys cys ala lys lys arg leu ala pro ser val leu arg ser leu ser glu val ser trp pro gln cys tyr his ser
9541/3181                                9571/3191                                9601/3201
TGG TTA TGG CAG CAC TGC ATA ATT CTC TTA CTG TCA TGC CAT CCG TAA GAT GCT TTT CTG TGA CTG GTG AGT ACT CAA CCA AGT CAT TCT
trp leu trp gln his cys ile ile leu leu leu ser cys his pro OCH asp ala phe leu OPA leu val ser thr gln pro ser his ser
9631/3211                                9661/3221                                9691/3231
GAG AAT AGT GTA TGC GGC GAC CGA GTT GCT CTT GCC CGG CGT CAA TAC GGG ATA ATA CCG CGC CAC ATA GCA GAA CTT TAA AAG TGC TCA
glu asn ser val cys gly asp arg val ala leu ala arg arg gln tyr gly ile ile pro arg his ile ala glu leu OCH lys cys ser
9721/3241                                9751/3251                                9781/3261
TCA TTG GAA AAC GTT CTT CGG GGC GAA AAC TCT CAA GGA TCT TAC CGC TGT TGA GAT CCA GTT CGA TGT AAC CCA CTC GTG CAC CCA ACT
ser leu glu asn val leu arg gly glu asn ser gln gly ser tyr arg cys OPA asp pro val arg cys asn pro leu val his pro thr
9811/3271                                9841/3281                                9871/3291
GAT CTT CAG CAT CTT TTA CTT TCA CCA GCG TTT CTG GGT GAG CAA AAA CAG GAA GGC AAA ATG CCG CAA AAA AGG GAA TAA GGG CGA CAC
asp leu gln his leu leu leu ser pro ala phe leu gly glu gln lys gln glu gly lys met pro gln lys arg glu OCH gly arg his
9901/3301                                9931/3311                                9961/3321
GGA AAT GTT GAA TAC TCA TAC TCT TCC TTT TTC AAT ATT ATT GAA GCA TTT ATC AGG GTT ATT GTC TCA TGA GCG GAT ACA TAT TTG AAT
gly asn val glu tyr ser tyr ser ser phe phe asn ile ile glu ala phe ile arg val ile val ser OPA ala asp thr tyr leu asn
9991/3331                                10021/3341                               10051/3351
GTA TTT AGA AAA ATA AAC AAA TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT
val phe arg lys ile asn lys AMB gly phe arg ala his phe pro glu lys cys his leu thr ser lys lys pro leu leu ser OPA his
10081/3361                               10111/3371                               10141/3381
TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TCG CGC GTT TCG GTG ATG ACG GTG AAA ACC TCT GAC ACA TGC AGC TCC CGG
OCH pro ile lys ile gly val ser arg gly pro phe val ser arg val ser val met thr val lys thr ser asp thr cys ser ser arg
10171/3391                               10201/3401                               10231/3411
AGA CGG TCA CAG CTT GTC TGT AAG CGG ATG CCG GGA GCA GAC AAG CCC GTC AGG GCG CGT CAG CGG GTG TTG GCG GGT GTC GGG GCT GGC
arg arg ser gln leu val cys lys arg met pro gly ala asp lys pro val arg ala arg gln arg val leu ala gly val gly ala gly
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Fig 1E

pACgp67-ScFv461 -> 1-phase Translation 16/01/1 22:01:48 Page 5

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10261/3421                                    10291/3431                                    10321/3441
TTA ACT ATG CGG CAT CAG AGC AGA TTG TAC TGA GAG TGC ACC ATA TGC GGT GTG AAA TAC CGC ACA GAT GCG TAA GGA GAA AAT ACC GCA
leu thr met arg his gln ser arg leu tyr OPA glu cys thr ile cys gly val lys tyr arg thr asp ala OCH gly glu asn thr ala
10351/3451                                    10381/3461                                    10411/3471
TCA GGC GCC ATT CGC CAT TCA GGC TGC GCA ACT GTT GGG AAG GGC GAT CGG TGC GGG CCT CTT CGC TAT TAC GCC AGC TGG CGA AAG GGG
ser gly ala ile arg his ser gly cys ala thr val gly lys gly asp arg cys gly pro leu arg tyr tyr ala ser trp arg lys gly
10441/3481                                    10471/3491                                    10501/3501
GAT GTG CTG CAA GGC GAT TAA GTT GGG TAA CGC CAG GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GTG CC
asp val leu gln gly asp OCH val gly OCH arg gln gly phe pro ser his asp val val lys arg arg pro val
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Fig 2

DNA Strider™ 1.2 ### Mardi 16 janvier 2001 21:58:26

pACgp67-ScFv350 -> 1-phase Translation

DNA sequence 10511 b.p. AAGCTTTACTCG ... ACGGCCAGTGCC linear

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1/1                                          31/11                                        61/21
AAG CTT TAC TCG TAA AGC GAG TTG AAG GAT CAT ATT TAG TTG CGT TTA TGA GAT AAG ATT GAA AGC ACG TGT AAA ATG TTT CCC GCG CGT
lys leu tyr ser OCH ser glu leu lys asp his ile AMB leu arg leu OPA asp lys ile glu ser thr cys lys met phe pro ala arg
91/31                                        121/41                                       151/51
TGG CAC AAC TAT TTA CAA TGC GGC CAA GTT ATA AAA GAT TCT AAT CTG ATA TGT TTT AAA ACA CCT TTG CGG CCC GAG TTG TTT GCG TAC
trp his asn tyr leu gln cys gly gln val ile lys asp ser asn leu ile cys phe lys thr pro leu arg pro glu leu phe ala tyr
181/61                                       211/71                                       241/81
GTG ACT AGC GAA GAA GAT GTG TGG ACC GCA GAA CAG ATA GTA AAA CAA AAC CCT AGT ATT GGA GCA ATA ATC GAT TTA ACC AAC ACG TCT
val thr ser glu glu asp val trp thr ala glu gln ile val lys gln asn pro ser ile gly ala ile ile asp leu thr asn thr ser
271/91                                       301/101                                      331/111
AAA TAT TAT GAT GGT GTG CAT TTT TTG CGG GCG GGC CTG TTA TAC AAA AAA ATT CAA GTA CCT GGC CAG ACT TTG CCG CCT GAA AGC ATA
lys tyr tyr asp gly val his phe leu arg ala gly leu leu tyr lys lys ile gln val pro gly gln thr leu pro pro glu ser ile
361/121                                      391/131                                      421/141
GTT CAA GAA TTT ATT GAC ACG GTA AAA GAA TTT ACA GAA AAG TGT CCC GGC ATG TTG GTG GGC GTG CAC TGC ACA CAC GGT ATT AAT CGC
val gln glu phe ile asp thr val lys glu phe thr glu lys cys pro gly met leu val gly val his cys thr his gly ile asn arg
451/151                                      481/161                                      511/171
ACC GGT TAC ATG GTG TGC AGA TAT TTA ATG CAC ACC CTG GGT ATT GCG CCG CAG GAA GCC ATA GAT AGA TTC GAA AAA GCC AGA GGT CAC
thr gly tyr met val cys arg tyr leu met his thr leu gly ile ala pro gln glu ala ile asp arg phe glu lys ala arg gly his
541/181                                      571/191                                      601/201
AAA ATT GAA AGA CAA AAT TAC GTT CAA GAT TTA TTA ATT TAA TTA ATA TTA TTT GCA TTC TTT AAC AAA TAC TTT ATC CTA TTT TCA AAT
lys ile glu arg gln asn tyr val gln asp leu leu ile OCH leu ile leu phe ala phe phe asn lys tyr phe ile leu phe ser asn
631/211                                      661/221                                      691/231
TGT TGC GCT TCT TCC AGC GAA CCA AAA CTA TGC TTC GCT TGC TCC GTT TAG CTT GTA GCC GAT CAG TGG CGT TGT TCC AAT CGA CGG TAG
cys cys ala ser ser ser glu pro lys leu cys phe ala cys ser val AMB leu val ala asp gln trp arg cys ser asn arg arg AMB
721/241                                      751/251                                      781/261
GAT TAG GCC GGA TAT TCT CCA CCA CAA TGT TGG CAA CGT TGA TGT TAC GTT TAT GCT TTT GGT TTT CCA CGT ACG TCT TTT GGC CGG TAA
asp AMB ala gly tyr ser pro pro gln cys trp gln arg OPA cys tyr val tyr ala phe gly phe pro arg thr ser phe gly arg OCH
811/271                                      841/281                                      871/291
TAG CCG TAA ACG TAG TGC CGT CGC GCG TCA CGC ACA ACA CCG GAT GTT TGC GCT TGT CCG CGG GGT ATT GAA CCG CGC GAT CCG ACA AAT
AMB pro OCH thr AMB cys arg arg ala ser arg thr thr pro asp val cys ala cys pro arg gly ile glu pro arg asp pro thr asn
901/301                                      931/311                                      961/321
CCA CCA CTT TGG CAA CTA AAT CGG TGA CCT GCG CGT CTT TTT TCT GCA TTA TTT CGT CTT TCT TTT GCA TGG TTT CCT GGA AGC CGG TGT
pro pro leu trp gln leu asn arg OPA pro ala arg leu phe ser ala leu phe arg leu ser phe ala trp phe pro gly ser arg cys
991/331                                      1021/341                                     1051/351
ACA TGC GGT TTA GAT CAG TCA TGA CGC GCG TGA CCT GCA AAT CTT TGG CCT CGA TCT GCT TGT CCT TGA TGG CAA CGA TGC GTT CAA TAA
thr cys gly leu asp gln ser OPA arg ala OPA pro ala asn leu trp pro arg ser ala cys pro OPA trp gln arg cys val gln OCH
1081/361                                     1111/371                                     1141/381
ACT CTT GTT TTT TAA CAA GTT CCT CGG TTT TTT GCG CCA CCA CCG CTT GCA GCG CGT TTG TGT GCT CGG TGA ATG TCG CAA TCA GCT TAG
thr leu val phe OCH gln val pro arg phe phe ala pro pro pro leu ala ala arg leu cys ala arg OPA met ser gln ser ala AMB
1171/391                                     1201/401                                     1231/411
TCA CCA ACT GTT TGC TCT CCT CCT CCC GTT GTT TGA TCG CGG GAT CGT ACT TGC CGG TGC AGA GCA CTT GAG GAA TTA CTT CTT CTA AAA
ser pro thr val cys ser pro pro pro val val OPA ser arg asp arg thr cys arg cys arg ala leu glu glu leu leu leu leu lys
1261/421                                     1291/431                                     1321/441
GCC ATT CTT GTA ATT CTA TGG CGT AAG GCA ATT TGG ACT TCA TAA TCA GCT GAA TCA CGC CGG ATT TAG TAA TGA GCA CTG TAT GCG GCT
ala ile leu val ile leu trp arg lys ala ile trp thr ser OCH ser ala glu ser arg arg ile AMB OCH OPA ala leu tyr ala ala
1351/451                                     1381/461                                     1411/471
GCA AAT ACA GCG GGT CGC CCC TTT TCA CGA CGC TGT TAG AGG TAG GGC CCC CAT TTT GGA TGG TCT GCT CAA ATA ACG ATT TGT ATT TAT
ala asn thr ala gly arg pro phe ser arg arg cys AMB arg AMB gly pro his phe gly trp ser ala gln ile thr ile cys ile tyr
1441/481                                     1471/491                                     1501/501
TGT CTA CAT GAA CAC GTA TAG CTT TAT CAC AAA CTG TAT ATT TTA AAC TGT TAG CGA CGT CCT TGG CCA CGA ACC GGA CCT GTT GGT CGC
cys leu his glu his val AMB leu tyr his lys leu tyr ile leu asn cys AMB arg arg pro trp pro arg thr gly pro val gly arg
1531/511                                     1561/521                                     1591/531
GCT CTA GCA CGT ACC GCA GGT TGA ACG TAT CTT CTC CAA ATT TAA ATT CTC CAA TTT TAA CGC GAG CCA TTT TGA TAC ACG TGT GTC GAT
ala leu ala arg thr ala gly OPA thr tyr leu leu gln ile OCH ile leu gln phe OCH arg glu pro phe OPA tyr thr cys val asp
1621/541                                     1651/551                                     1681/561
TTT GCA ACA ACT ATT GTT TTT TAA CGC AAA CTA AAC TTA TTG TGG TAA GCA ATA ATT AAA TAT GGG GGA ACA TGC GCC GCT ACA ACA CTC
phe ala thr thr ile val phe OCH arg lys leu asn leu leu trp OCH ala ile ile lys tyr gly gly thr cys ala ala thr thr leu
1711/571                                     1741/581                                     1771/591
GTC GTT ATG AAC GCA GAC GGC GCC GGT CTC GGC GCA AGC GGC TAA AAC GTG TTG CGC GTT CAA CGC GGC AAA CAT CGC AAA AGC CAA TAG
val val met asn ala asp gly ala gly leu gly ala ser gly OCH asn val leu arg val gln arg gly lys his arg lys ser gln AMB
1801/601                                     1831/611                                     1861/621
TAC AGT TTT GAT TTG CAT ATT AAC GGC GAT TTT TTA AAT TAT CTT ATT TAA TAA ATA GTT ATG ACG CCT ACA ACT CCC CGC CCG CGT TGA
tyr ser phe asp leu his ile asn gly asp phe leu asn tyr leu ile OCH OCH ile val met thr pro thr thr pro arg pro arg OPA
1891/631                                     1921/641                                     1951/651
CTC GCT GCA CCT CGA GCA GTT CGT TGA CGC CTT CCT CCG TGT GGC CGA ACA CGT CGA GCG GGT GGT CGA TGA CCA GCG GCG TGC CGC ACG
leu ala ala pro arg ala val arg OPA arg leu pro pro cys gly arg thr arg arg ala gly gly arg OPA pro ala ala cys arg thr
1981/661                                     2011/671                                     2041/681
CGA CGC ACA AGT ATC TGT ACA CCG AAT GAT CGT CGG GCG AAG GCA CGT CGG CCT CCA AGT GGC AAT ATT GGC AAA TTC GAA AAT ATA TAC
arg arg thr ser ile cys thr pro asn asp arg arg ala lys ala arg arg pro pro ser gly asn ile gly lys phe glu asn ile tyr
2071/691                                     2101/701                                     2131/711
AGT TGG GTT GTT TGC GCA TAT CTA TCG TGG CGT TGG GCA TGT ACG TCC GAA CGT TGA TTT GCA TGC AAG CCG AAA TTA AAT CAT TGC GAT
ser trp val val cys ala tyr leu ser trp arg trp ala cys thr ser glu arg OPA phe ala cys lys pro lys leu asn his cys asp
2161/721                                     2191/731                                     2221/741
TAG TGC GAT TAA AAC GTT GTA CAT CCT CGC TTT TAA TCA TGC CGT CGA TTA AAT CGC GCA ATC GAG TCA AGT GAT CAA AGT GTG GAA TAA
AMB cys asp OCH asn val val his pro arg phe OCH ser cys arg arg leu asn arg ala ile glu ser ser asp gln ser val glu OCH
2251/751                                     2281/761                                     2311/771
TGT TTT CTT TGT ATT CCC GAG TCA AGC GCA GCG CGT ATT TTA ACA AAC TAG CCA TCT TGT AAG TTA GTT TCA TTT AAT GCA ACT TTA TCC
cys phe leu cys ile pro glu ser ser ala ala arg ile leu thr asn AMB pro ser cys lys leu val ser phe asn ala thr leu ser
2341/781                                     2371/791                                     2401/801
AAT AAT ATA TTA TGT ATC GCA CGT CAA GAA TTA ACA ATG CGC CCG TTG TCG CAT CTC AAC ACG ACT ATG ATA GAG ATC AAA TAA AGC GCG
asn asn ile leu cys ile ala arg gln glu leu thr met arg pro leu ser his leu asn thr thr met ile glu ile lys OCH ser ala
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pACgp67-ScFv350 -> 1-phase Translation   Fig. 2B   16/01/1 21:58:26 Page 2

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2431/811                                     2461/821                                     2491/831
AAT TAA ATA GCT TGC GAC GCA ACG TGC ACG ATC TGT GCA CGC GTT CCG GCA CGA GCT TTG ATT GTA ATA AGT TTT TAC GAA GCG ATG ACA
asn OCH ile ala cys asp ala thr cys thr ile cys ala arg val pro ala arg ala leu ile val ile ser phe tyr glu ala met thr
2521/841                                     2551/851                                     2581/861
TGA CCC CCG TAG TGA CAA CGA TCA CGC CCA AAA GAA CTG CCG ACT ACA AAA TTA CCG AGT ATG TCG GTG ACG TTA AAA CTA TTA AGC CAT
OPA pro pro AMB OPA gln arg ser arg pro lys glu leu pro thr thr lys leu pro ser met ser val thr leu lys leu leu ser his
2611/871                                     2641/881                                     2671/891
CCA ATC GAC CGT TAG TCG AAT CAG GAC CGC TGG TGC GAG AAG CCG CGA AGT ATG GCG AAT GCA TCG TAT AAC GTG TGG AGT CCG CTC ATT
pro ile asp arg AMB ser asn gln asp arg trp cys glu lys pro arg ser met ala asn ala ser tyr asn val trp ser pro leu ile
2701/901                                     2731/911                                     2761/921
AGA GCG TCA TGT TTA GAC AAG AAA GCT ACA TAT TTA ATT GAT CCC GAT GAT TTT ATT GAT AAA TTG ACC CTA ACT CCA TAC ACG GTA TTC
arg ala ser cys leu asp lys lys ala thr tyr leu ile asp pro asp asp phe ile asp lys leu thr leu thr pro tyr thr val phe
2791/931                                     2821/941                                     2851/951
TAC AAT GGC GGG GTT TTG GTC AAA ATT TCC GGA CTG CGA TTG TAC ATG CTG TTA ACG GCT CCG CCC ACT ATT AAT GAA ATT AAA AAT TCC
tyr asn gly gly val leu val lys ile ser gly leu arg leu tyr met leu leu thr ala pro pro thr ile asn glu ile lys asn ser
2881/961                                     2911/971                                     2941/981
AAT TTT AAA AAA CGC AGC AAG AGA AAC ATT TGT ATG AAA GAA TGC GTA GAA GGA AAG AAA AAT GTC GTC GAC ATG CTG AAC AAC AAG ATT
asn phe lys lys arg ser lys arg asn ile cys met lys glu cys val glu gly lys lys asn val val asp met leu asn asn lys ile
2971/991                                     3001/1001                                    3031/1011
AAT ATG CCT CCG TGT ATA AAA AAA ATA TTG AAC GAT TTG AAA GAA AAC AAT GTA CCG CGC GGC GGT ATG TAC AGG AAG AGG TTT ATA CTA
asn met pro pro cys ile lys lys ile leu asn asp leu lys glu asn asn val pro arg gly gly met tyr arg lys arg phe ile leu
3061/1021                                    3091/1031                                    3121/1041
AAC TGT TAC ATT GCA AAC GTG GTT TCG TGT GCC AAG TGT GAA AAC CGA TGT TTA ATC AAG GCT CTG ACG CAT TTC TAC AAC CAC GAC TCC
asn cys tyr ile ala asn val val ser cys ala lys cys glu asn arg cys leu ile lys ala leu thr his phe tyr asn his asp ser
3151/1051                                    3181/1061                                    3211/1071
AAG TGT GTG GGT GAA GTC ATG CAT CTT TTA ATC AAA TCC CAA GAT GTG TAT AAA CCA CCA AAC TGC CAA AAA ATG AAA ACT GTC GAC AAG
lys cys val gly glu val met his leu leu ile lys ser gln asp val tyr lys pro pro asn cys gln lys met lys thr val asp lys
3241/1081                                    3271/1091                                    3301/1101
CTC TGT CCG TTT GCT GGC AAC TGC AAG GGT CTC AAT CCT ATT TGT AAT TAT TGA ATA ATA AAA CAA TTA TAA ATG CTA AAT TTG TTT TTT
leu cys pro phe ala gly asn cys lys gly leu asn pro ile cys asn tyr OPA ile ile lys gln leu OCH met leu asn leu phe phe
3331/1111                                    3361/1121                                    3391/1131
ATT AAC GAT ACA AAC CAA ACG CAA CAA GAA CAT TTG TAG TAT TAT CTA TAA TTG AAA ACG CGT AGT TAT AAT CGC TGA GGT AAT ATT TAA
ile asn asp thr asn gln thr gln gln glu his leu AMB tyr tyr leu OCH leu lys thr arg ser tyr asn arg OPA gly asn ile OCH
3421/1141                                    3451/1151                                    3481/1161
AAT CAT TTT CAA ATG ATT CAC AGT TAA TTT GCG ACA ATA TAA TTT TAT TTT CAC ATA AAC TAG ACG CCT TGT CGT CTT CTT CTT CGT ATT
asn his phe gln met ile his ser OCH phe ala thr ile OCH phe tyr phe his ile asn AMB thr pro cys arg leu leu leu arg ile
3511/1171                                    3541/1181                                    3571/1191
CCT TCT CTT TTT CAT TTT TCT CCT CAT AAA AAT TAA CAT AGT TAT TAT CGT ATC CAT ATA TGT ATC TAT CGT ATA GAG TAA ATT TTT TGT
pro ser leu phe his phe ser pro his lys asn OCH his ser tyr tyr arg ile his ile cys ile tyr arg ile glu OCH ile phe cys
3601/1201                                    3631/1211                                    3661/1221
TGT CAT AAA TAT ATA TGT CTT TTT TAA TGG GGT GTA TAG TAC CGC TGC GCA TAG TTT TTC TGT AAT TTA CAA CAG TGC TAT TTT CTG GTA
cys his lys tyr ile cys leu phe OCH trp gly val AMB tyr arg cys ala AMB phe phe cys asn leu gln gln cys tyr phe leu val
3691/1231                                    3721/1241                                    3751/1251
GTT CTT CGG AGT GTG TTG CTT TAA TTA TTA AAT TTA TAT AAT CAA TGA ATT TGG GAT CGT CGG TTT TGT ACA ATA TGT TGC CGG CAT AGT
val leu arg ser val leu leu OCH leu leu asn leu tyr asn gln OPA ile trp asp arg arg phe cys thr ile cys cys arg his ser
3781/1261                                    3811/1271                                    3841/1281
ACG CAG CTT CTT CTA GTT CAA TTA CAC CAT TTT TTA GCA GCA CCG GAT TAA CAT AAC TTT CCA AAA TGT TGT ACG AAC CGT TAA ACA AAA
thr gln leu leu leu val gln leu his his phe leu ala ala pro asp OCH his asn phe pro lys cys cys thr asn arg OCH thr lys
3871/1291                                    3901/1301                                    3931/1311
ACA GTT CAC CTC CCT TTT CTA TAC TAT TGT CTG CGA GCA GTT GTT TGT TGT TAA AAA TAA CAG CCA TTG TAA TGA GAC GCA CAA ACT AAT
thr val his leu pro phe leu tyr tyr cys leu arg ala val val cys cys OCH lys OCH gln pro leu OCH OPA asp ala gln thr asn
3961/1321                                    3991/1331                                    4021/1341
ATC ACA AAC TGG AAA TGT CTA TCA ATA TAT AGT TGC TGA TAC CAT GGA GAT AAT TAA AAT GAT AAC CAT CTC GCA AAT AAA TAA GTA TTT
ile thr asn trp lys cys leu ser ile tyr ser cys OPA tyr his gly asp asn OCH asn asp asn his leu ala asn lys OCH val phe
                                                                                                                 -> Pgp67
4051/1351                                    4081/1361                                    4111/1371
TAC TGT TTT CGT AAC AGT TTT GTA ATA AAA AAA CCT ATA AAT ATT CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CGC GGA TCT ATG CTA
tyr cys phe arg asn ser phe val ile lys lys pro ile asn ile pro asp tyr ser tyr arg pro thr ile gly arg gly ser met leu
4141/1381                                    4171/1391                                    4201/1401
CTA GTA AAT CAG TCA CAC CAA GGC TTC AAT AAG GAA CAC ACA AGC AAG ATG GTA AGC GCT ATT GTT TTA TAT GTG CTT TTG GCG GCG GCG
leu val asn gln ser his gln gly phe asn lys glu his thr ser lys met val ser ala ile val leu tyr val leu leu ala ala ala
4231/1411                                    4261/1421  Tag HiS     Facteur Xa          4291/1431  -> ScFv 350 = VH
GCG CAT TCT GCC TTT GCG GCG GAT CTT gga tcc CAT CAT CAC CAC CAC CAC att gaa gga aga GAA TTC CAG GTC CAA CTG CAG CAG TCT
ala his ser ala phe ala ala asp leu gly ser his his his his his his ile glu gly arg glu phe gln val gln leu gln gln ser
4321/1441                                    4351/1451                                    4381/1461
GGG GCT GAA CTG GCA AAA CCT GGG GCC TCA GTG AAG CTG TCC TGC AAG GCT TCT GGC CAC ACC TTT ACT AGC TAC TGG ATG CAC TGG GTA
gly ala glu leu ala lys pro gly ala ser val lys leu ser cys lys ala ser gly his thr phe thr ser tyr trp met his trp val
4411/1471                                    4441/1481                                    4471/1491
AAA CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT GGA TAC ATT AAT CTT AGC AGT GGT TAT ATT AAG TAC AAT CAG GAG TTC AAG GAC AAG
lys gln arg pro gly gln gly leu glu trp ile gly tyr ile asn leu ser ser gly tyr ile lys tyr asn gln glu phe lys asp lys
4501/1501                                    4531/1511                                    4561/1521
GCC ACA TTG ACT GCA GAC AAA TCC TCC AAC ACA GCC TAC ATG CAT CTG AGC AGC CTG ACA TAT GAG GAC TCT GCA GTC TAT TAC TGT GCA
ala thr leu thr ala asp lys ser ser asn thr ala tyr met his leu ser ser leu thr tyr glu asp ser ala val tyr tyr cys ala
                                                                                                       peptide de
4591/1531                                    4621/1541                                    4651/1551
AGG GCA GCT CAG GCT ACG ACC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC
arg ala ala gln ala thr thr phe asp tyr trp gly gln gly thr thr leu thr val ser ser gly gly gly gly ser gly gly gly gly
                                                                                                       jonction
4681/1561             -> : VL                4711/1571                                    4741/1581
TCT GGC GGT GGC GGA TCG GAC ATT GTG ATG ATC CAG TCT CAC AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG GTC AGC ATC ACC TGC AAG
ser gly gly gly gly ser asp ile val met ile gln ser his lys phe met ser thr ser val gly asp arg val ser ile thr cys lys
4771/1591                                    4801/1601                                    4831/1611
GCC AGT CAG GAT GTG AGT ACT GCT GTA GCC TGG TAT CAA CAA AAA CCA GGG CAA TCT CCT AAA CTA CTG ATT TAC TGG GCA TCC ACC CGG
ala ser gln asp val ser thr ala val ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp ala ser thr arg
4861/1621                                    4891/1631                                    4921/1641
CAC ACT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TAT ACT CTC ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA
his thr gly val pro asp arg phe thr gly ser gly ser gly thr asp tyr thr leu thr ile ser ser val gln ala glu asp leu ala
4951/1651                                    4981/1661                                    5011/1671
CTT TAT TAC TGT CAG CAA CAT TAT AGC ACT CCT CCG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT CCC GGG gga tgt TAA Stop
leu tyr tyr cys gln gln his tyr ser thr pro pro thr phe gly gly gly thr lys leu glu ile lys arg ala pro gly gly cys OCH
                                                                                                      2gly 1Cys
                                                                                                fin scFv
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: CDR des régions variables VH et V

Fig. 2C

pA Cgp67-ScFv350 -> 1-phase Translation 16/01/1 21:58:26 Page

5041/1681 5071/1691 5101/1701
aga tct gat ccT TTC CTG GGA CCC GGC AAG AAC CAA AAA CTC ACT CTC TTC AAG GAA ATC CGT AAT GTT AAA CCC GAC ACG ATG AAG CTT
arg ser asp pro phe leu gly pro gly lys asn gln lys leu thr leu phe lys glu ile arg asn val lys pro asp thr met lys leu
5131/1711 5161/1721 5191/1731
GTC GTT GGA TGG AAA GGA AAA GAG TTC TAC AGG GAA ACT TGG ACC CGC TTC ATG GAA GAC AGC TTC CCC ATT GTT AAC GAC CAA GAA GTG
val val gly trp lys gly lys glu phe tyr arg glu thr trp thr arg phe met glu asp ser phe pro ile val asn asp gln glu val
5221/1741 5251/1751 5281/1761
ATG GAT GTT TTC CTT GTT GTC AAC ATG CGT CCC ACT AGA CCC AAC CGT TGT TAC AAA TTC CTG GCC CAA CAC GCT CTG CGT TGC GAC CCC
met asp val phe leu val val asn met arg pro thr arg pro asn arg cys tyr lys phe leu ala gln his ala leu arg cys asp pro
5311/1771 5341/1781 5371/1791
GAC TAT GTA CCT CAT GAC GTG ATT AGG ATC GTC GAG CCT TCA TGG GTG GGC AGC AAC AAC GAG TAC CGC ATC AGC CTG GCT AAG AAG GGC
asp tyr val pro his asp val ile arg ile val glu pro ser trp val gly ser asn asn glu tyr arg ile ser leu ala lys lys gly
5401/1801 5431/1811 5461/1821
GGC GGC TGC CCA ATA ATG AAC CTT CAC TCT GAG TAC ACC AAC TCG TTC GAA CAG TTC ATC GAT CGT GTC ATC TGG GAG AAC TTC TAC AAG
gly gly cys pro ile met asn leu his ser glu tyr thr asn ser phe glu gln phe ile asp arg val ile trp glu asn phe tyr lys
5491/1831 5521/1841 5551/1851
CCC ATC GTT TAC ATC GGT ACC GAC TCT GCT GAA GAG GAG GAA ATT CTC CTT GAA GTT TCC CTG GTG TTC AAA GTA AAG GAG TTT GCA CCA
pro ile val tyr ile gly thr asp ser ala glu glu glu glu ile leu leu glu val ser leu val phe lys val lys glu phe ala pro
5581/1861 5611/1871 5641/1881
GAC GCA CCT CTG TTC ACT GGT CCG GCG TAT TAA AAC ACG ATA CAT TGT TAT TAG TAC ATT TAT TAA GCG CTA GAT TCT GTG CGT TGT TGA
asp ala pro leu phe thr gly pro ala tyr OCH asn thr ile his cys tyr AMB tyr ile tyr OCH ala leu asp ser val arg cys OPA
5671/1891 5701/1901 5731/1911
TTT ACA GAC AAT TGT TGT ACG TAT TTT AAT AAT TCA TTA AAT TTA TAA TCT TTA GGG TGG TAT GTT AGA GCG AAA ATC AAA TGA TTT TCA
phe thr asp asn cys cys thr tyr phe asn asn ser leu asn leu OCH ser leu gly trp tyr val arg ala lys ile lys OPA phe ser
5761/1921 5791/1931 5821/1941
GCG TCT TTA TAT CTG AAT TTA AAT ATT AAA TCC TCA ATA GAT TTG TAA AAT AGG TTT CGA TTA GTT TCA AAC AAG GGT TGT TTT TCC GAA
ala ser leu tyr leu asn leu asn ile lys ser ser ile asp leu OCH asn arg phe arg leu val ser asn lys gly cys phe ser glu
5851/1951 5881/1961 5911/1971
CCG ATG GCT GGA CTA TCT AAT GGA TTT TCG CTC AAC GCC ACA AAA CTT GCC AAA TCT TGT AGC AGC AAT CTA GCT TTG TCG ATA TTC GTT
pro met ala gly leu ser asn gly phe ser leu asn ala thr lys leu ala lys ser cys ser ser asn leu ala leu ser ile phe val
5941/1981 5971/1991 6001/2001
TGT GTT TTG TTT TGT AAT AAA GGT TCG ACG TCG TTC AAA ATA TTA TGC GCT TTT GTA TTT CTT TCA TCA CTG TCG TTA GTG TAC AAT TGA
cys val leu phe cys asn lys gly ser thr ser phe lys ile leu cys ala phe val phe leu ser ser leu ser leu val tyr asn OPA
6031/2011 6061/2021 6091/2031
CTC GAC GTA AAC ACG TTA AAT AAA GCT TGG ACA TAT TTA ACA TCG GGC GTG TTA GCT TTA TTA GGC CGA TTA TCG TCG TCG TCC CAA CCC
leu asp val asn thr leu asn lys ala trp thr tyr leu thr ser gly val leu ala leu leu gly arg leu ser ser ser ser gln pro
6121/2041 6151/2051 6181/2061
TCG TCG TTA GAA GTT GCT TCC GAA GAC GAT TTT GCC ATA GCC ACA CGA CGC CTA TTA ATT GTG TCG GCT AAC ACG TCC GCG ATC AAA TTT
ser ser leu glu val ala ser glu asp asp phe ala ile ala thr arg arg leu leu ile val ser ala asn thr ser ala ile lys phe
6211/2071 6241/2081 6271/2091
GTA GTT GAG CTT TTT GGA ATT ATT TCT GAT TGC GGG CGT TTT TGG GCG GGT TTC AAT CTA ACT GTG CCC GAT TTT AAT TCA GAC AAC ACG
val val glu leu phe gly ile ile ser asp cys gly arg phe trp ala gly phe asn leu thr val pro asp phe asn ser asp asn thr
6301/2101 6331/2111 6361/2121
TTA GAA AGC GAT GGT GCA GGC GGT GGT AAC ATT TCA GAC GGC AAA TCT ACT AAT GGC GGC GGT GGT GGA GCT GAT GAT AAA TCT ACC ATC
leu glu ser asp gly ala gly gly gly asn ile ser asp gly lys ser thr asn gly gly gly gly gly ala asp asp lys ser thr ile
6391/2131 6421/2141 6451/2151
GGT GGA GGC GCA GGC GGG GCT GGC GGC GGA GGC GGA GGC GGA GGT GGT GGC GGT GAT GCA GAC GGC GGT TTA GGC TCA AAT GTC TCT TTA
gly gly gly ala gly gly ala gly gly gly gly gly gly gly gly gly gly gly asp ala asp gly gly leu gly ser asn val ser leu
6481/2161 6511/2171 6541/2181
GGC AAC ACA GTC GGC ACC TCA ACT ATT GTA CTG GTT TCG GGC GCC GTT TTT GGT TTG ACC GGT CTG AGA CGA GTG CGA TTT TTT TCG TTT
gly asn thr val gly thr ser thr ile val leu val ser gly ala val phe gly leu thr gly leu arg arg val arg phe phe ser phe
6571/2191 6601/2201 6631/2211
CTA ATA GCT TCC AAC AAT TGT TGT CTG TCG TCT AAA GGT GCA GCG GGT TGA GGT TCC GTC GGC ATT GGT GGA GCG GGC GGC AAT TCA GAC
leu ile ala ser asn asn cys cys leu ser ser lys gly ala ala gly OPA gly ser val gly ile gly gly ala gly gly asn ser asp
6661/2221 6691/2231 6721/2241
ATC GAT GGT GGT GGT GGT GGT GGA GGC GCT GGA ATG TTA GGC ACG GGA GAA GGT GGT GGC GGC GGT GCC GCT GGT ATA ATT TGT TCT GGT
ile asp gly gly gly gly gly gly gly ala gly met leu gly thr gly glu gly gly gly gly gly ala ala gly ile ile cys ser gly
6751/2251 6781/2261 6811/2271
TTA GTT TGT TCG CGC ACG ATT GTG GGC ACC GGC GCA GGC GCC GCT GGC TGC ACA ACG GAA GGT CGT CTG CTT CGA GGC AGC GCT TGG GGT
leu val cys ser arg thr ile val gly thr gly ala gly ala ala gly cys thr thr glu gly arg leu leu arg gly ser ala trp gly
6841/2281 6871/2291 6901/2301
GGT GGC AAT TCA ATA TTA TAA TTG GAA TAC AAA TCG TAA AAA TCT GCT ATA AGC ATT GTA ATT TCG CTA TCG TTT ACC GTG CCG ATA TTT
gly gly asn ser ile leu OCH leu glu tyr lys ser OCH lys ser ala ile ser ile val ile ser leu ser phe thr val pro ile phe
6931/2311 6961/2321 6991/2331
AAC AAC CGC TCA ATG TAA GCA ATT GTA TTG TAA AGA GAT TGT CTC AAG CTC CGC ACG CCG ATA ACA AGC CTT TTC ATT TTT ACT ACA GCA
asn asn arg ser met OCH ala ile val leu OCH arg asp cys leu lys leu arg thr pro ile thr ser leu phe ile phe thr thr ala
7021/2341 7051/2351 7081/2361
TTG TAG TGG CGA GAC ACT TCG CTG TCG TCG ACG TAC ATG TAT GCT TTG TTG TCA AAA ACG TCG TTG GCA AGC TTT AAA ATA TTT AAA AGA
leu AMB trp arg asp thr ser leu ser ser thr tyr met tyr ala leu leu ser lys thr ser leu ala ser phe lys ile phe lys arg
7111/2371 7141/2381 7171/2391
ACA TCT CTG TTC AGC ACC ACT GTG TTG TCG TAA ATG TTG TTT TTG ATA ATT TGC GCT TCC GCA GTA TCG ACA CGT TCA AAA AAT TGA TGC
thr ser leu phe ser thr thr val leu ser OCH met leu phe leu ile ile cys ala ser ala val ser thr arg ser lys asn OPA cys
7201/2401 7231/2411 7261/2421
GCA TCA ATT TTG TTG TTC CTA TTA TTG AAT AAA TAA GAT TGT ACA GAT TCA TAT CTA CGA TTC GTC ATG GCC ACC ACA AAT GCT ACG CTG
ala ser ile leu leu phe leu leu leu asn lys OCH asp cys thr asp ser tyr leu arg phe val met ala thr thr asn ala thr leu
7291/2431 7321/2441 7351/2451
CAA ACG CTG GTA CAA TTT TAC GAA AAC TGC AAA AAC GTC AAA ACT CGG TAT AAA ATA ATC AAC GGG CGC TTT GGC AAA ATA TCT ATT TTA
gln thr leu val gln phe tyr glu asn cys lys asn val lys thr arg tyr lys ile ile asn gly arg phe gly lys ile ser ile leu
7381/2461 7411/2471 7441/2481
TCG CAC AAG CCC ACT AGC AAA TTG TAT TTG CAG AAA ACA ATT TCG GCG CAC AAT TTT AAC GCT GAC GAA ATA AAA GTT CAC CAG TTA ATG
ser his lys pro thr ser lys leu tyr leu gln lys thr ile ser ala his asn phe asn ala asp glu ile lys val his gln leu met
7471/2491 7501/2501 7531/2511
AGC GAC CAC CCA AAT TTT ATA AAA ATC TAT TTT AAT CAC GGT TCC ATC AAC AAC CAA GTG ATC GTG ATG GAC TAC ATT GAC TGT CCC GAT
ser asp his pro asn phe ile lys ile tyr phe asn his gly ser ile asn asn gln val ile val met asp tyr ile asp cys pro asp
7561/2521 7591/2531 7621/2541
TTA TTT GAA ACA CTA CAA ATT AAA GGC GAG CTT TCG TAC CAA CTT GTT AGC AAT ATT ATT AGA CAG CTG TGT GAA GCG CTC AAC GAT TTG
leu phe glu thr leu gln ile lys gly glu leu ser tyr gln leu val ser asn ile ile arg gln leu cys glu ala leu asn asp leu

pACgp67-ScFv350 -> 1-phase Translation

Fig 2D

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7651/2551                                 7681/2561                                 7711/2571
CAC AAG CAC AAT TTC ATA CAC AAC GAC ATA AAA CTC GAA AAT GTC TTA TAT TTC GAA GCA CTT GAT CGC GTG TAT GTT TGC GAT TAC GGA
his lys his asn phe ile his asn asp ile lys leu glu asn val leu tyr phe glu ala leu asp arg val tyr val cys asp tyr gly
7741/2581                                 7771/2591                                 7801/2601
TTG TGC AAA CAC GAA AAC TCA CTT AGC GTG CAC GAC GGC ACG TTG GAG TAC TTT AGT CCG GAA AAA ATT CGA CAC ACA ACT ATG CAC GTT
leu cys lys his glu asn ser leu ser val his asp gly thr leu glu tyr phe ser pro glu lys ile arg his thr thr met his val
7831/2611                                 7861/2621                                 7891/2631
TCG TTT GAC TGG TAC GCG GCG TGT TAA CAT ACA AGT TGC TAA CCG Gcg gtt cGT AAT CAT GGT CAT AGC TGT TTC CTG TGT GAA ATT GTT
ser phe asp trp tyr ala ala cys OCH his thr ser cys OCH pro ala val arg asn his gly his ser cys phe leu cys glu ile val
7921/2641                                 7951/2651                                 7981/2661
ATC CGC TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GCA TAA AGT GTA AAG CCT GGG GTG CCT AAT GAG TGA GCT AAC TCA CAT TAA TTG
ile arg ser gln phe his thr thr tyr glu pro glu ala OCH ser val lys pro gly val pro asn glu OPA ala asn ser his OCH leu
8011/2671                                 8041/2681                                 8071/2691
CGT TGC GCT CAC TGC CCG CTT TCC AGT CGG GAA ACC TGT CGT GCC AGC TGC ATT AAT GAA TCG GCC AAC GCG CGG GGA GAG GCG GTT TGC
arg cys ala his cys pro leu ser ser arg glu thr cys arg ala ser cys ile asn glu ser ala asn ala arg gly glu ala val cys
8101/2701                                 8131/2711                                 8161/2721
GTA TTG GGC GCT CTT CCG CTT CCT CGC TCA CTG ACT CGC TGC GCT CGG TCG TTC GGC TGC GGC GAG CGG TAT CAG CTC ACT CAA AGG CGG
val leu gly ala leu pro leu pro arg ser leu thr arg cys ala arg ser phe gly cys gly glu arg tyr gln leu thr gln arg arg
8191/2731                                 8221/2741                                 8251/2751
TAA TAC GGT TAT CCA CAG AAT CAG GGG ATA ACG CAG GAA AGA ACA TGT GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA AGG CCG
OCH tyr gly tyr pro gln asn gln gly ile thr gln glu arg thr cys glu gln lys ala ser lys arg pro gly thr val lys arg pro
8281/2761                                 8311/2771                                 8341/2781
CGT TGC TGG CGT TTT TCC ATA GGC TCC GCC CCC CTG ACG AGC ATC ACA AAA ATC GAC GCT CAA GTC AGA GGT GGC GAA ACC CGA CAG GAC
arg cys trp arg phe ser ile gly ser ala pro leu thr ser ile thr lys ile asp ala gln val arg gly gly glu thr arg gln asp
8371/2791                                 8401/2801                                 8431/2811
TAT AAA GAT ACC AGG CGT TTC CCC CTG GAA GCT CCC TCG TGC GCT CTC CTG TTC CGA CCC TGC CGC TTA CCG GAT ACC TGT CCG CCT TTC
tyr lys asp thr arg arg phe pro leu glu ala pro ser cys ala leu leu phe arg pro cys arg leu pro asp thr cys pro pro phe
8461/2821                                 8491/2831                                 8521/2841
TCC CTT CGG GAA GCG TGG CGC TTT CTC ATA GCT CAC GCT GTA GGT ATC TCA GTT CGG TGT AGG TCG TTC GCT CCA AGC TGG GCT GTG TGC
ser leu arg glu ala trp arg phe leu ile ala his ala val gly ile ser val arg cys arg ser phe ala pro ser trp ala val cys
8551/2851                                 8581/2861                                 8611/2871
ACG AAC CCC CCG TTC AGC CCG ACC GCT GCG CCT TAT CCG GTA ACT ATC GTC TTG AGT CCA ACC CGG TAA GAC ACG ACT TAT CGC CAC TGG
thr asn pro pro phe ser pro thr ala ala pro tyr pro val thr ile val leu ser pro thr arg OCH asp thr thr tyr arg his trp
8641/2881                                 8671/2891                                 8701/2901
CAG CAG CCA CTG GTA ACA GGA TTA GCA GAG CGA GGT ATG TAG GCG GTG CTA CAG AGT TCT TGA AGT GGT GGC CTA ACT ACG GCT ACA CTA
gln gln pro leu val thr gly leu ala glu arg gly met AMB ala val leu gln ser ser OPA ser gly gly leu thr thr ala thr leu
8731/2911                                 8761/2921                                 8791/2931
GAA GGA CAG TAT TTG GTA TCT GCG CTC TGC TGA AGC CAG TTA CCT TCG GAA AAA GAG TTG GTA GCT CTT GAT CCG GCA AAC AAA CCA CCG
glu gly gln tyr leu val ser ala leu cys OPA ser gln leu pro ser glu lys glu leu val ala leu asp pro ala asn lys pro pro
8821/2941                                 8851/2951                                 8881/2961
CTG GTA GCG GTG GTT TTT TTG TTT GCA AGC AGC AGA TTA CGC GCA GAA AAA AAG GAT CTC AAG AAG ATC CTT TGA TCT TTT CTA CGG GGT
leu val ala val val phe leu phe ala ser ser arg leu arg ala glu lys lys asp leu lys lys ile leu OPA ser phe leu arg gly
8911/2971                                 8941/2981                                 8971/2991
CTG ACG CTC AGT GGA ACG AAA ACT CAC GTT AAG GGA TTT TGG TCA TGA GAT TAT CAA AAA GGA TCT TCA CCT AGA TCC TTT TAA ATT AAA
leu thr leu ser gly thr lys thr his val lys gly phe trp ser OPA asp tyr gln lys gly ser ser pro arg ser phe OCH ile lys
9001/3001                                 9031/3011                                 9061/3021
AAT GAA GTT TTA AAT CAA TCT AAA GTA TAT ATG AGT AAA CTT GGT CTG ACA GTT ACC AAT GCT TAA TCA GTG AGG CAC CTA TCT CAG CGA
asn glu val leu asn gln ser lys val tyr met ser lys leu gly leu thr val thr asn ala OCH ser val arg his leu ser gln arg
9091/3031                                 9121/3041                                 9151/3051
TCT GTC TAT TTC GTT CAT CCA TAG TTG CCT GAC TCC CCG TCG TGT AGA TAA CTA CGA TAC GGG AGG GCT TAC CAT CTG GCC CCA GTG CTG
ser val tyr phe val his pro AMB leu pro asp ser pro ser cys arg OCH leu arg tyr gly arg ala tyr his leu ala pro val leu
9181/3061                                 9211/3071                                 9241/3081
CAA TGA TAC CGC GAG ACC CAC GCT CAC CGG CTC CAG ATT TAT CAG CAA TAA ACC AGC CAG CCG GAA GGG CCG AGC GCA GAA GTG GTC CTG
gln OPA tyr arg glu thr his ala his arg leu gln ile tyr gln gln OCH thr ser gln pro glu gly pro ser ala glu val val leu
9271/3091                                 9301/3101                                 9331/3111
CAA CTT TAT CCG CCT CCA TCC AGT CTA TTA ATT GTT GCC GGG AAG CTA GAG TAA GTA GTT CGC CAG TTA ATA GTT TGC GCA ACG TTG TTG
gln leu tyr pro pro pro ser ser leu leu ile val ala gly lys leu glu OCH val val arg gln leu ile val cys ala thr leu leu
9361/3121                                 9391/3131                                 9421/3141
CCA TTG CTA CAG GCA TCG TGG TGT CAC GCT CGT CGT TTG GTA TGG CTT CAT TCA GCT CCG GTT CCC AAC GAT CAA GGC GAG TTA CAT GAT
pro leu leu gln ala ser trp cys his ala arg arg leu val trp leu his ser ala pro val pro asn asp gln gly glu leu his asp
9451/3151                                 9481/3161                                 9511/3171
CCC CCA TGT TGT GCA AAA AAG CGG TTA GCT CCT TCG GTC CTC CGA TCG TTG TCA GAA GTA AGT TGG CCG CAG TGT TAT CAC TCA TGG TTA
pro pro cys cys ala lys lys arg leu ala pro ser val leu arg ser leu ser glu val ser trp pro gln cys tyr his ser trp leu
9541/3181                                 9571/3191                                 9601/3201
TGG CAG CAC TGC ATA ATT CTC TTA CTG TCA TGC CAT CCG TAA GAT GCT TTT CTG TGA CTG GTG AGT ACT CAA CCA AGT CAT TCT GAG AAT
trp gln his cys ile ile leu leu leu ser cys his pro OCH asp ala phe leu OPA leu val ser thr gln pro ser his ser glu asn
9631/3211                                 9661/3221                                 9691/3231
AGT GTA TGC GGC GAC CGA GTT GCT CTT GCC CGG CGT CAA TAC GGG ATA ATA CCG CGC CAC ATA GCA GAA CTT TAA AAG TGC TCA TCA TTG
ser val cys gly asp arg val ala leu ala arg arg gln tyr gly ile ile pro arg his ile ala glu leu OCH lys cys ser ser leu
9721/3241                                 9751/3251                                 9781/3261
GAA AAC GTT CTT CGG GGC GAA AAC TCT CAA GGA TCT TAC CGC TGT TGA GAT CCA GTT CGA TGT AAC CCA CTC GTG CAC CCA ACT GAT CTT
glu asn val leu arg gly glu asn ser gln gly ser tyr arg cys OPA asp pro val arg cys asn pro leu val his pro thr asp leu
9811/3271                                 9841/3281                                 9871/3291
CAG CAT CTT TTA CTT TCA CCA GCG TTT CTG GGT GAG CAA AAA CAG GAA GGC AAA ATG CCG CAA AAA AGG GAA TAA GGG CGA CAC GGA AAT
gln his leu leu leu ser pro ala phe leu gly glu gln lys gln glu gly lys met pro gln lys arg glu OCH gly arg his gly asn
9901/3301                                 9931/3311                                 9961/3321
GTT GAA TAC TCA TAC TCT TCC TTT TTC AAT ATT ATT GAA GCA TTT ATC AGG GTT ATT GTC TCA TGA GCG GAT ACA TAT TTG AAT GTA TTT
val glu tyr ser tyr ser ser phe phe asn ile ile glu ala phe ile arg val ile val ser OPA ala asp thr tyr leu asn val phe
9991/3331                                 10021/3341                                10051/3351
AGA AAA ATA AAC AAA TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT
arg lys ile asn lys AMB gly phe arg ala his phe pro glu lys cys his leu thr ser lys lys pro leu leu ser OPA his OCH pro
10081/3361                                10111/3371                                10141/3381
ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TCG CGC GTT TCG GTG ATG ACG GTG AAA ACC TCT GAC ACA TGC AGC TCC CGG AGA CGG
ile lys ile gly val ser arg gly pro phe val ser arg val ser val met thr val lys thr ser asp thr cys ser ser arg arg arg
10171/3391                                10201/3401                                10231/3411
TCA CAG CTT GTC TGT AAG CGG ATG CCG GGA GCA GAC AAG CCC GTC AGG GCG CGT CAG CGG GTG TTG GCG GGT GTC GGG GCT GGC TTA ACT
ser gln leu val cys lys arg met pro gly ala asp lys pro val arg ala arg gln arg val leu ala gly val gly ala gly leu thr
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pACgp67-ScFv350 -> 1-phase Translation   Fig 2E   16/01/1 21:58:26 Page 5

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10261/3421                                    10291/3431                                    10321/3441
ATG CGG CAT CAG AGC AGA TTG TAC TGA GAG TGC ACC ATA TGC GGT GTG AAA TAC CGC ACA GAT GCG TAA GGA GAA AAT ACC GCA TCA GGC
met arg his gln ser arg leu tyr OPA glu cys thr ile cys gly val lys tyr arg thr asp ala OCH gly glu asn thr ala ser gly
10351/3451                                    10381/3461                                    10411/3471
GCC ATT CGC CAT TCA GGC TGC GCA ACT GTT GGG AAG GGC GAT CGG TGC GGG CCT CTT CGC TAT TAC GCC AGC TGG CGA AAG GGG GAT GTG
ala ile arg his ser gly cys ala thr val gly lys gly asp arg cys gly pro leu arg tyr tyr ala ser trp arg lys gly asp val
10441/3481                                    10471/3491                                    10501/3501
CTG CAA GGC GAT TAA GTT GGG TAA CGC CAG GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GTG CC
leu gln gly asp OCH val gly OCH arg gln gly phe pro ser his asp val val lys arg arg pro val
```

Figure 3 : clonetherap.99B3

VH sequence :
GAGGTGAAGCTTCTCCAGTCTGGAGGTGGCCTGGTGCAGCCTGGAGGATCCCTGA
AACTCTCCTGTGCAGCCTCAGGAATCGATTTTAGTAGATACTGGATGAGTTGGGT
TCGGCGGGCTCCAGGGAAAGGACTAGAATGGATTGGAGAAATTAATCCAGATAG
CAGTACAATAAACTATGCACCATCTCTAAAGGATAAATTCATCATCTCCAGAGAC
AACGCCAAAAATACGCTGTACCTGCAAATGAGCAAAGTGAGATCTGAGGACACA
GCCCTTTATTACTGTGCAAGAGGACTGGGACAGAACTTTGACTACTGGGGCCAAG
GCACCACTCTCACAGTCTCCTCA

VL sequence :
GATATTGTGATGACGCAGGCTGCATTCTCCAATCCAGTCACTCTTGGAACATCAG
CTTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTACATAGTAATGGCATCACTTAT
TTGTATTGGTATCTGCAGAAGCCAGGCCAGTCTCCTCAGCTCCTGATTTATCAGAT
GTCCAACCTTGCCTCAGGAGTCCCAGACAGGTTCAGTAGCAGTGGGTCAGGAACT
GATTTCACACTGAGAATCAGCAGAGTGGAGGCTGAGGATGTGGGTGTTTATTACT
GTGCTCAAAATCTAGAACTTCCGTGGACGTTCGGTGGAGGCACCAAGCTGGAAAT
CAAA

Figure 4 : clonetherap.88E10

VH sequence :
GAGGTGAAGCTGGTGGAGTCTGGAGGAGGCTTGGTACAGCCTGGGGGTTCTCTG
AGTCTCTCCTGTGCAGCTTCTGGATTCACCTTCACTGATTACTCCATGAACTGGGT
CCGCCAGCCTCCAGGGAAGACACTTGAGTGGTTGGCTTTTATTAGAAACAAAGCT
AATGGTTACACAGCAGAGTACAGTGCATCTGTGAAGGGTCGGTTCTCCATCTCCA
GAGATAATTCCCAAAGCATCCTCTATCTTCAAATGAATGCCCTGAGAGCTGAGGA
CAGTGCCACTTATTACTGTGCAAGGGGATGGTATGCTATGGACTACTGGGGTCAA
GGAACCTCAGTCACCGTCTCCTCA

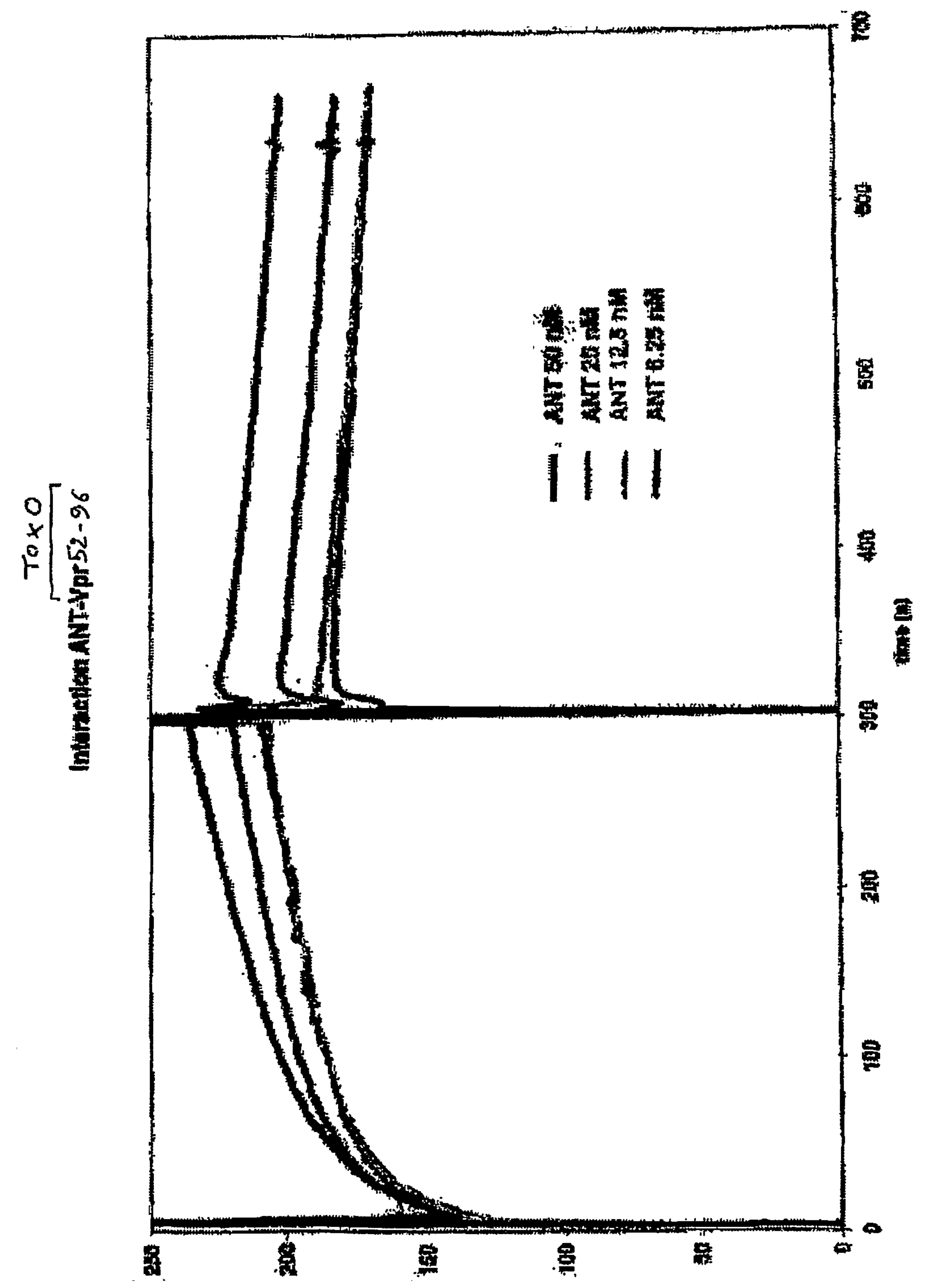
VL sequence :

Figure 5 : clonetherap.152C3

VH sequence :
GAGGTTCTGCTGCAGCAGTCTGTGGCAGAGCTTGTGAGGCCAGGGGCCTCAGTCA
AGTTGTCCTGCATAGTTTCTGACTTCAACATTAAACACACCTATATGCACTGGGTG
AAACAGAGGCCTGATCAGGGCCTGGAGTGGATTGGAAGGATTGATCCTGCGAAT
GGTAAAACTATATATGCCCCGACGTTCCAGGGCAAGGCCACTATAACTGCGGACA
CATCCTCCAACACAGCCTACCTGCATTTCAGCAGCCTGACATCTGAGGACGCTGC
CATCTATTACTGTGCTAGAGCTGGGGCTGGCTACTTTGACTACTGGGGCCAAGGC
ACCACTCTCACAGTCTCCTCA

VL sequence :
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAG
TCAGTTTCTCCTGCAGGGCCAGTCAGAACATTGGCACAAGTATTTACTGGTATCA
GCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAGTATGTTTCTGAGTCTATC
TCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGATCAGGGACAGAGTTTACTCTTA
GCATCAACAGTGTGGAGTCTGAAGATATTGCAGATTATTACTGTCAACAAAGTCA
TAGTTGGCCGCTCACGTTCGTGCTGGGACCAAGCTGGAGCTGAAA

Fig 6


ToxO
Interaction ANT-Vpr52-96
ANT 50 nM
ANT 25 nM
ANT 12,5 nM
ANT 6,25 nM
0
50
100
150
200
250
0
100
200
300
400
500
600
700

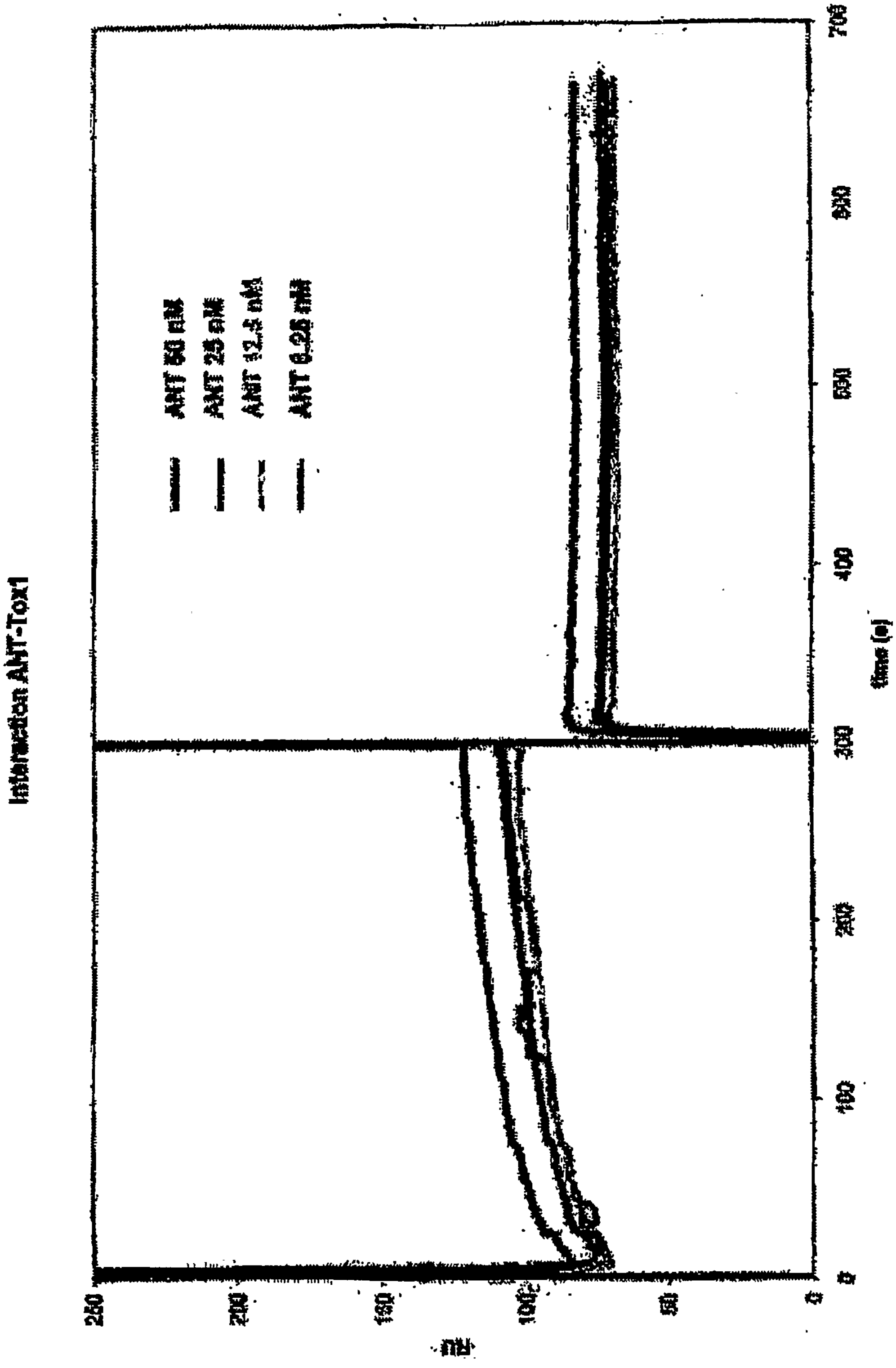

Interaction ANT-Tox1
ANT 50 nM
ANT 25 nM
ANT 12.5 nM
ANT 6.25 nM
RU
time (s)

Fig. 7

fig. 8

Tox0
Interaction VDAC-Vpr52-96

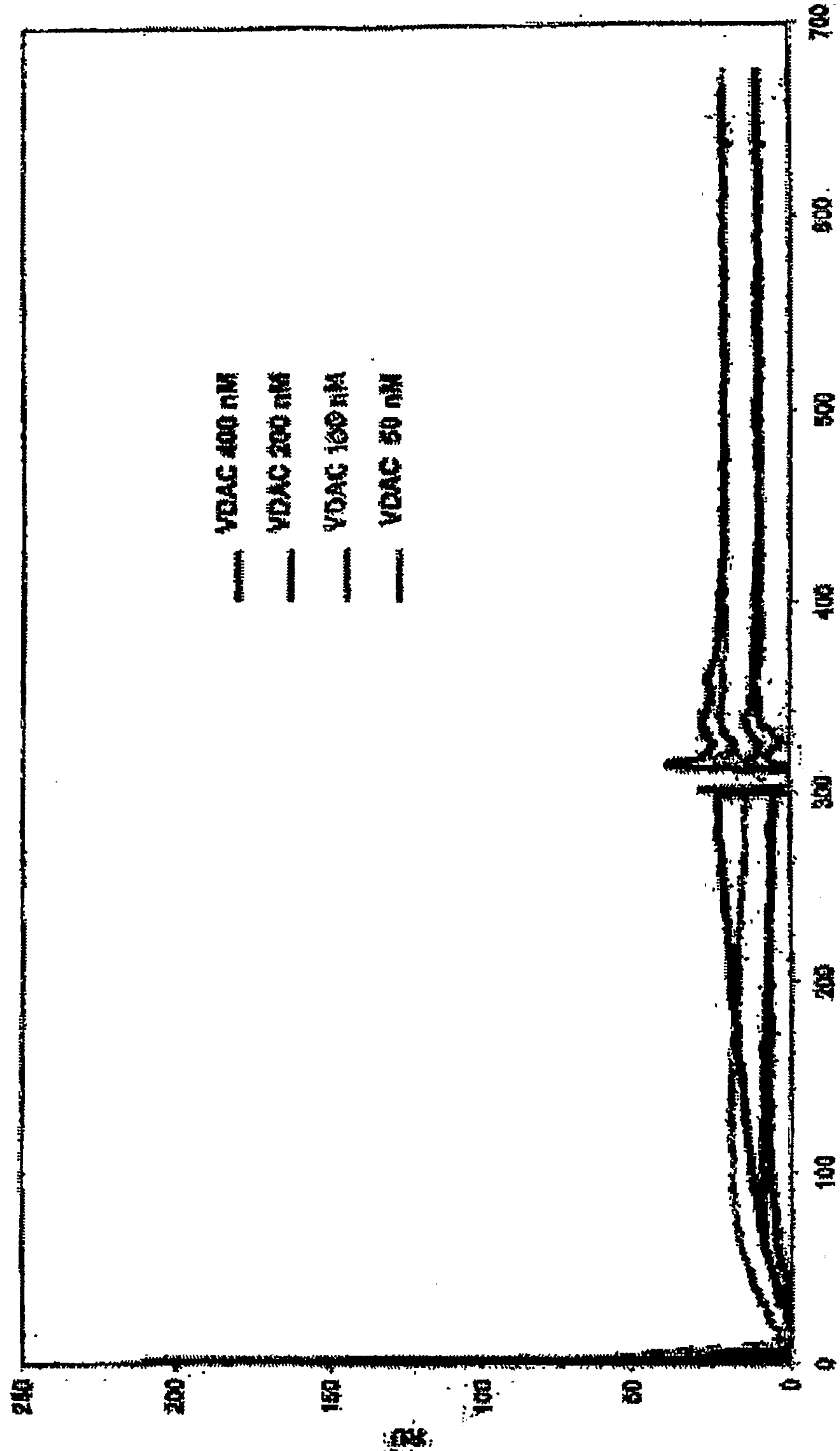


Fig 9

Interaction VDAC-Tox1

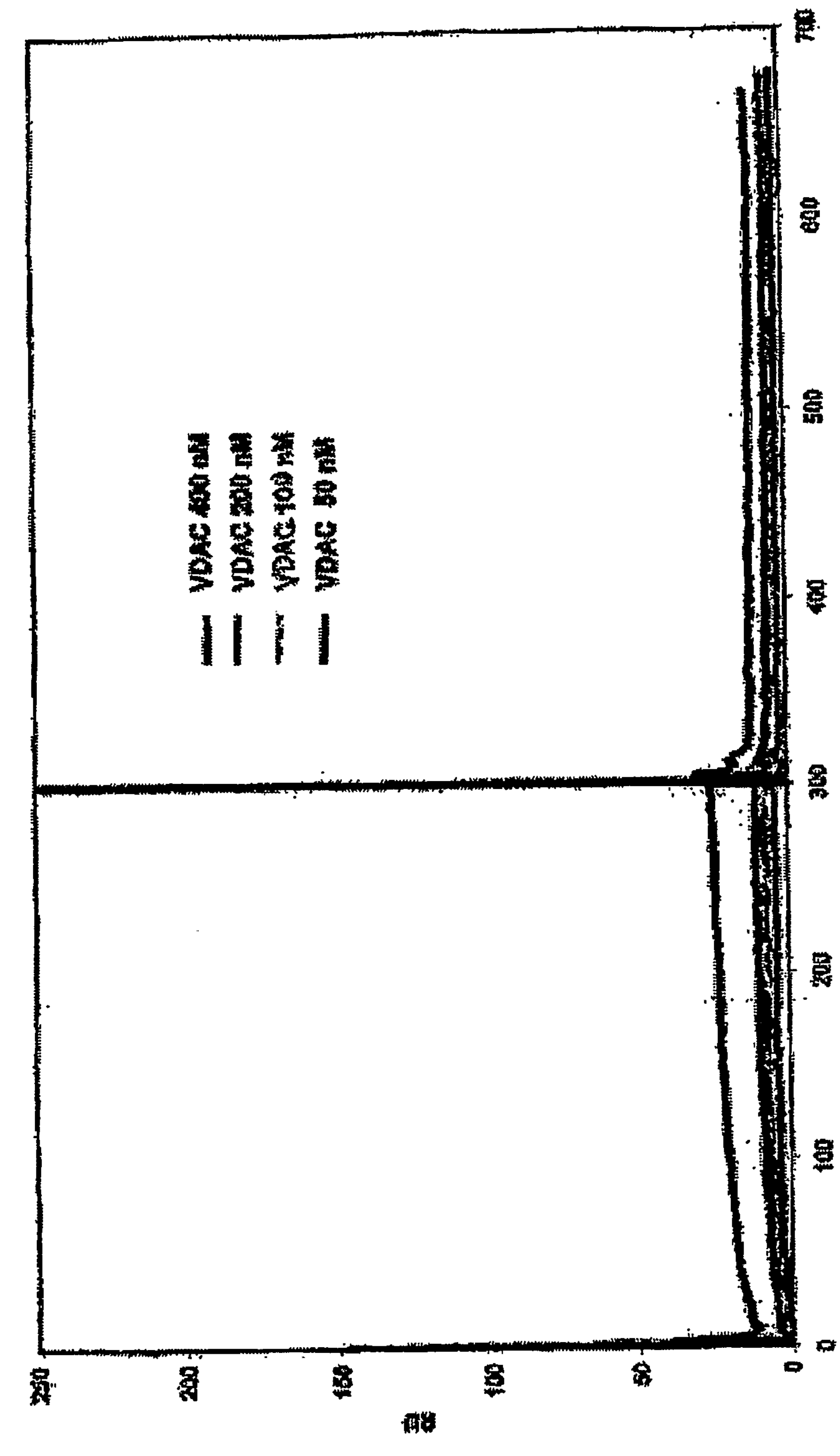
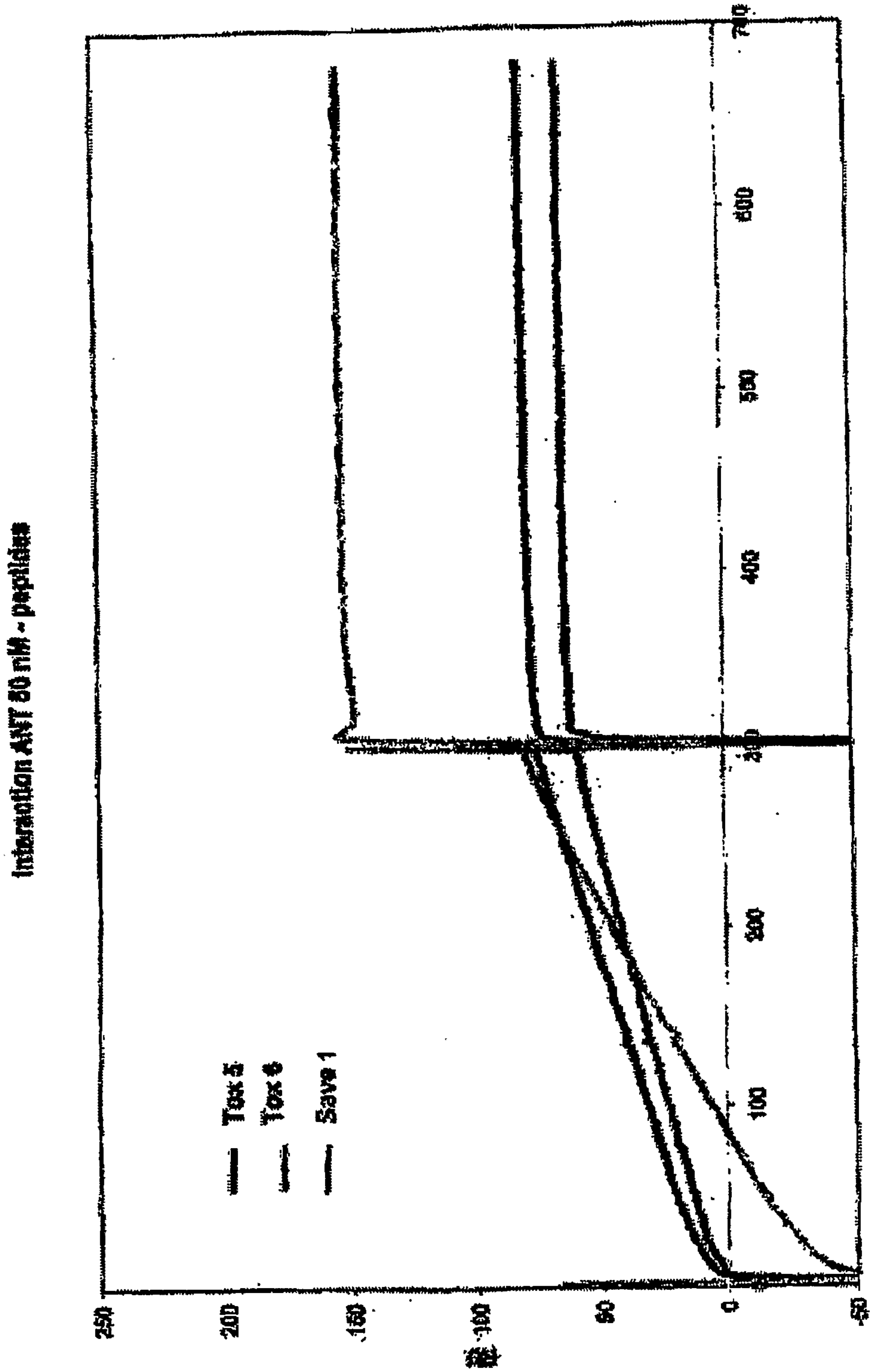


Fig. 10


Interaction ANT 50 nM - peptides
Tox 5
Tox 6
Save 1
time (s)
250
200
150
100
50
0
-50
100
200
300
400
500
600
700

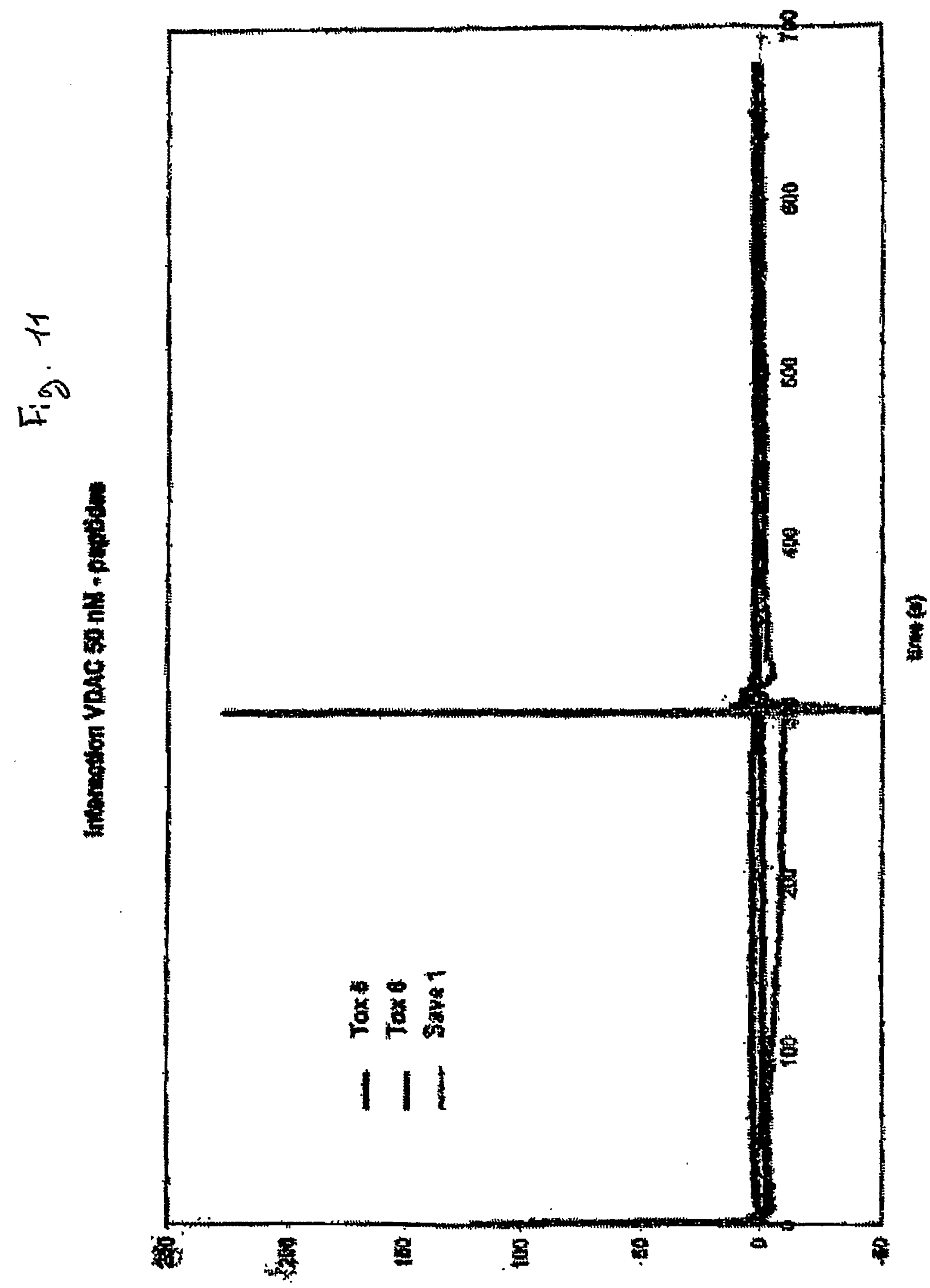


Fig. 11
Interaction VDAC 50 nM + peptides
Tox 5
Tox 6
Save 1
time (s)

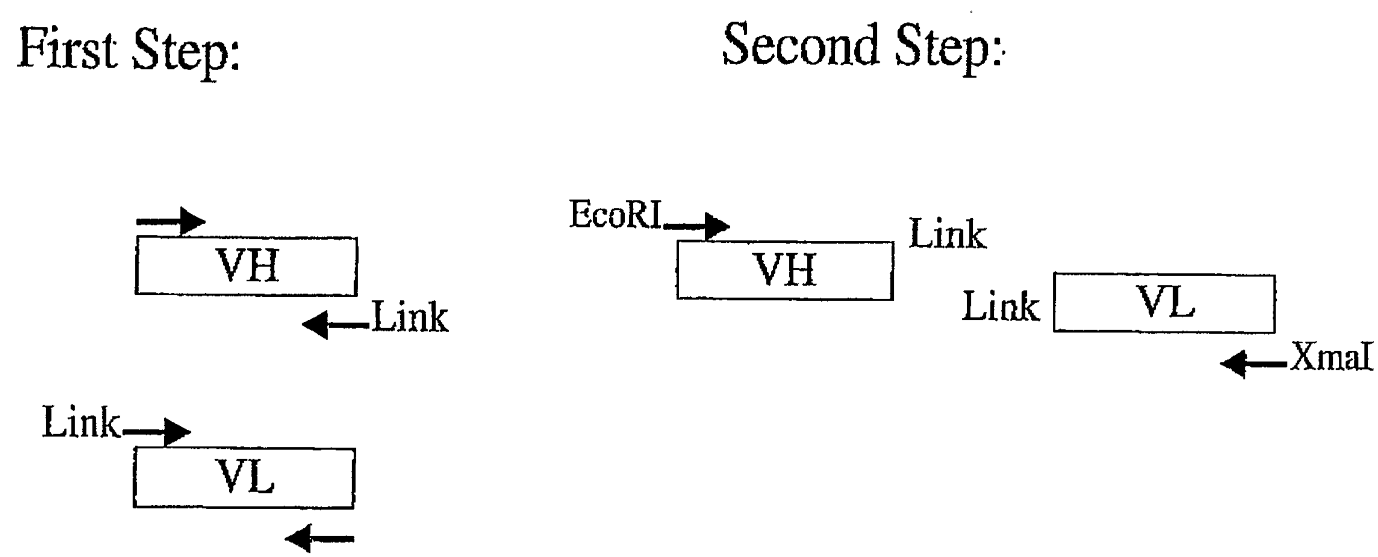


Figure 12: Obtention of the VH/VL chimeric DNA

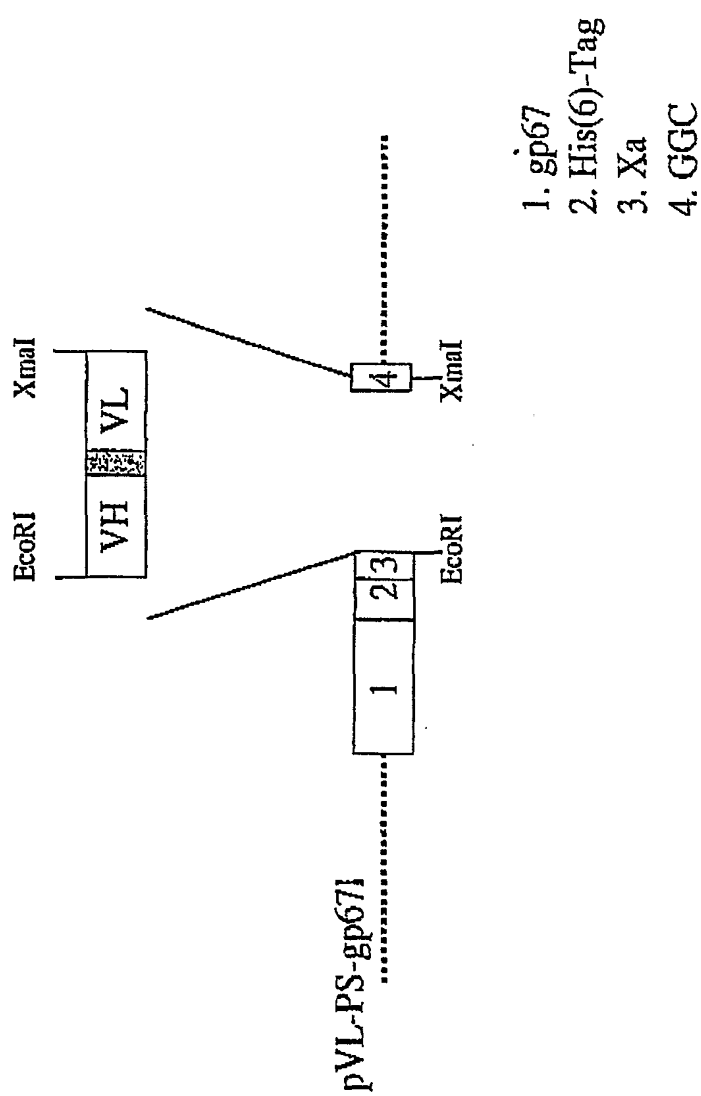


Figure 13: Map of the ScFv transfert vector